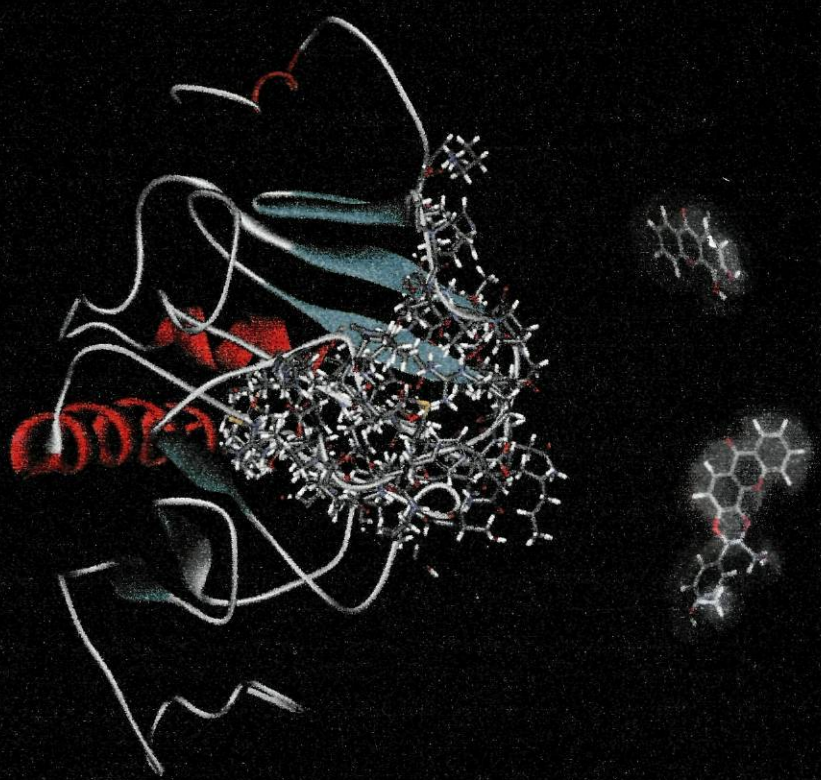
DERIVADOS DE XANTEN-9-ONA :

Síntese, Determinação Estrutural e Avaliação de Actividades Biológicas



Maria Emília da Silva Pereira Sousa

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Orientador :
Professora Catedrática Madalena Maria de Magalhães Pinto

Co-Orientadores :
Professor Doutor Carlos Manuel Magalhães Afonso
Professora Doutora Maria de São José Nascimento

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Resumo

Esta dissertação inclui a síntese e a determinação estrutural de doze xantonas oxigenadas simples e de cinco xantonolignóides, bem como a avaliação das respectivas actividades biológicas, inibição do crescimento de linhas celulares tumorais humanas e inibição da proliferação de linfócitos humanos *in vitro*, e modulação da quinase C de proteínas.

É descrito o planeamento e a síntese baseada em processos biomiméticos de cinco xantonolignóides: a *trans*-(±)-kielcorina C, a *cis*-(±)-kielcorina C*, a *trans*-(±)-kielcorina D*, a *trans*-(±)-isokielcorina D* e a *trans*-(±)-kielcorina E*. A metodologia de síntese baseou-se na reacção de acoplamento oxidativo de *orto*-di-hidroxixantonas com o álcool coniferílico na presença de um agente oxidante. A estereoquímica e a regioquímica do processo de síntese foi analisada por comparação de vários agentes oxidantes e por estudos computacionais. Foi desenvolvido um método de HPLC para a separação e quantificação dos derivados kielcorínicos. Foi efectuada a resolução analítica dos cinco pares de enantiómeros kielcorínicos, por HPLC, em diferentes fases estacionárias quirais. A coluna de *tris*-3,5-dimetilfenilcarbamato de amilose foi a escolhida para a resolução semi-preparativa das misturas racémicas *trans*-(±)-kielcorina C, *trans*-(±)-kielcorina D*, *trans*-(±)-isokielcorina D* e *trans*-(±)-kielcorina E*.

Foram sintetizadas as seguintes xantonas simples oxigenadas: 1-metoxi-, 1-hidroxi-, 2-metoxi-, 2-hidroxi-, 3-metoxi-, 3-hidroxi-, 1,2-dimetoxi-, 1,2-di-hidroxi-, 2,3-dimetoxi-, 2,3-di-hidroxi-, 3,4-dimetoxi- e 3,4-di-hidroxixantona. A metodologia de síntese destes compostos foi baseada na formação de intermediários ciclizáveis, do tipo benzofenona ou éter bifenílico, ou na reacção de Grover, Shah e Shah. Um novo método de síntese é descrito para xantonas 1,2-di-oxigenadas.

As estruturas dos derivados xantónicos e dos intermediários de síntese foram todas estabelecidas com base em métodos espectroscópicos (IV, UV, RMN de ^{1}H e ^{13}C, COSY, HETCOR, HMBC, HSQC, NOE e INEPT selectivo), EM de impacto electrónico e alta resolução e adicionalmente por cristalografia de raios X para os compostos 1,2-dimetoxixantona, 2-(3,4-dimetoxifenoxi)-benzoato de metilo e ácido 2-(3,4-dimetoxifenoxi)-benzóico.

Neste trabalho foi avaliada a actividade dos compostos sintetizados sobre o crescimento de três linhas celulares tumorais humanas, MCF-7 (adenocarcinoma da mama),

* Compostos descritos pela primeira vez.

TK-10 (carcinoma renal) e UACC-62 (melanoma) e ainda o efeito dos enantiómeros kielcorínicos isolados no crescimento da linha celular MCF-7. Foi também investigada a capacidade dos derivados xantónicos em inibir a proliferação de linfócitos periféricos humanos. A interacção destes compostos com as isoformas α, βI, δ, η e ζ da quinase C de proteínas foi também estudada.

Abstract

This thesis reports the synthesis and structure elucidation of twelve simple oxygenated xanthones and five xanthonolignoids, as well as the evaluation of their biological activities: *in vitro* inhibition of growth of human cancer cell lines and of proliferation of human lymphocytes, and modulation of protein kinase C.

The design and synthesis, based on a biomimetic pathway of five xanthonolignoids, *trans*-(±)-kielcorin C, *cis*-(±)-kielcorin C *, *trans*-(±)-kielcorin D *, *trans*-(±)-isokielcorin D*, and *trans*-(±)-kielcorin E *, are described.

The synthetic approach was based on the oxidative coupling reaction of *ortho*-dihydroxyxanthones with coniferyl alcohol in the presence of an oxidizing agent. The stereo- and regiochemistry of the synthetic process was analyzed by comparison of several oxidizing agents and by computational studies. The HPLC method for separation and quantification of kielcorin derivatives was developed. The analytical resolution of the five pairs of kielcorin enantiomers was performed by HPLC in different chiral stationary phases. The amylose *tris*-3,5-dimethylphenylcarbamate column was selected for semi-preparative resolution of the racemic mixtures: *trans*-(±)-kielcorin C *, *trans*-(±)-kielcorin D *, *trans*-(±)-isokielcorin D *, and *trans*-(±)-kielcorin E *.

The following oxygenated simple xanthones were synthesized: 1-methoxy -, 1-hydroxy -, 2-methoxy -, 2-hydroxy -, 3-methoxy -, 3-hydroxy -, 1,2-dimethoxy -, 1,2-dihydroxy -, 2,3-dimethoxy -, 2,3-dihydroxy -, 3,4-dimethoxy- and 3,4-dihydroxyxanthone. The synthetic approach of these compounds was based on the formation of cyclizable intermediates, benzophenone or biphenyl ether, or on the Grover, Shah and Shah reaction. A new synthetic method for 1,2-dioxygenated xanthones is also described.

The structures of xanthone derivatives and their synthetic intermediates were established by spectroscopic methods (IR, UV, NMR of ^{1}H and ^{13}C, COSY, HETCOR, HMBC, HSQC, NOE and selective INEPT), mass spectrometry, and additionally by X-ray crystallography for 1,2-dimethoxyxanthone, methyl 2-(3,4-dimethoxyphenoxy)benzoate and 2-(3,4-dimethoxyphenoxy)benzoic acid.

The antitumor activity of the compounds was evaluated by the *in vitro* growth inhibition of three human cancer cell lines: MCF-7 (breast adenocarcinoma), TK-10 (renal carcinoma), and UACC-62 (melanoma), whereas the effect of the isolated kielcorin enantiomers was done on the growth of cell line MCF-7. The xanthone derivatives were also

* Compounds described for the first time.

evaluated for their capacity to inhibit the proliferation of human peripheral lymphocytes as well as their interaction with the α, βI, δ, η, and ζ isoforms of protein kinase C.

Résumé

Cette dissertation inclut la synthèse et l' élucidation structurale de douze xanthones simples oxygénées et de cinq xanthonolignoides, aussi bien que l'évaluation des activités biologiques respectives, l'inhibition sur la croissance des lignées cellulaires néoplasiques humaines, l'inhibition de la prolifération des lymphocytes humains *in vitro*, et la modulation de la protéine kinase C.

Il y est décrit la planification et la synthèse basées sur des processus biomimetiques de cinq xantonolignoides : *trans*-(±)-kielcorine C, *cis*-(±)-kielcorine C *, *trans*-(±)-kielcorine D*, *trans*-(±)-isokielcorine D * et *trans*-(±)-kielcorine E *. La méthodologie de synthèse a été basée sur la réaction d'accouplement oxydatif du *ortho*-dihydroxyxanthones avec l'alcool coniferyl en présence d'un agent oxydant. La stereochimie et la regiochimie du processus de synthèse ont été analysées par comparaison de plusieurs agents oxydants et àtravers des études informatiques. Il a été développé une méthode de chromatographie liquide sous haute pression pour la séparation et la quantification des dérivés de kielcorines. La résolution analytique des cinq paires d'enantioméres de kielcorines a été exécutée, pour la chromatographie liquide sous haute pression, dans différentes phases stationnaires chirales. La colonne de *tris*-3,5-diméthylphénilcarbamate de amylose a été choisie pour la résolution semi-préparatoire du *trans*-(±)-kielcorine C *, *trans*-(±)-kielcorine D * et *trans*-(±)-kielcorine E *.

Il a été synthétisé les suivantes xanthones simples oxygénées: 1-méthoxy -, 1-hydroxy -, 2-méthoxy -, 2-hydroxy -, 3-méthoxy -, 3-hydroxy -, 1,2-diméthoxy -, 1,2-dihydroxy -, 2,3-diméthoxy -, 2,3-dihydroxy -, 3,4-diméthoxy- et 3,4-dihydroxyxanthone. La méthodologie de synthèse de ces composés a été basée sur la formation d'intermédiaires cyclizables, du type benzophenone ou éther de biphényles, ou sur la réaction de Grover, Shah et Shah. Une nouvelle méthode de synthèse est décrite pour les xanthones 1,2-dioxigénées.

Les structures des dérivés de xanthones et des intermédiaires de synthèse ont été toutes établies es ayant comme base des méthodes spectroscopiques (IR, UV, RMN de ^{1}H et ^{13}C, COSY, HETCOR, HMBC, HSQC, NOE et INEPT sélectif, spectrométrie de masse d'impact électronique et de haute résolution) et aussi par cristallographie des rayons X pour les composés 1,2-diméthoxyxanthone, méthyl 2-(3,4-diméthoxyphenoxy)-benzoate et 2-(3,4-diméthoxyphenoxy)-benzoique acide.

Dans ce travail il a été evalué l'activité des composés synthétisés sur la croissance des

* Substances décrites pour la primière fois.

trois lignées cellulaires néoplasiques humaines, MCF-7 (adénocarcinome mamelle), TK-10 (carcinome rénal) et UACC-62 (mélanome), et aussi l'effet des enantioméres de kielcorines isolés dans la croissance de la ligne cellulaire MCF-7. Il a été aussi étudié la capacité des dérivés de xanthones à empêcher la prolifération des lymphocytes périphériques humaines. Aussi que l'interaction de ces composés avec les isoformes, βI, δ, η et ζ de protéine kinase C.

CEQOFFUP	Centro de Estudos de Química Orgânica, Fitoquímica e Farmacologia da Universidade do Porto
COSY	*Correlated spectroscopy*
EM	Espectrometria de massa
HETCOR	*Heteronuclear chemical shift correlation*
HMBC	*Heteronuclear multiple bound correlation*
HPLC	Cromatografia líquida de alta eficiência
HSQC	*Heteronuclear single quantum correlation*
INEPT	*Insensitive nuclei enhancement by polarization transfer*
IV	Infravermelho
NOE	*Nuclear Overhauser effect*
PKC	Quinase C de proteínas
RMN	Ressonância magnética nuclear
UV	Ultravioleta

Organização e Objectivos da Tese

Ao longo da dissertação é remetida a leitura dos resultados e discussão para um conjunto de artigos científicos, já publicados ou em publicação, que fazem parte integrante deste trabalho e que se encontram em Anexos (pág. 94):

Anexo I Pinto, M. M. M.; Sousa, E. P. Natural and Synthetic Xanthonolignoids: Chemistry and Biological Activities. *Curr. Med. Chem.* **2003**, *10*, 1-12.

Anexo II Sousa, E. P.; Silva, A. M. S.; Pinto, M. M. M.; Pedro, M. M.; Cerqueira, F. A. M.; Nascimento, M. S. J. Isomeric Kielcorins and Dihydroxyxanthones: Synthesis, Structure Elucidation and Inhibitory Activities of Growth of Human Cancer Cell Lines and on the Proliferation of Human Lymphocytes *In Vitro*. *Helv. Chim. Acta* **2002**, *85*, 2862-2876.

Anexo III Sousa, M. E.; Afonso, M. M. C.; Pinto, M. M. M. Quantitative Analysis of Kielcorins in Biomimetic Synthesis by Liquid Chromatography/UV Detection. *J. Liq. Chrom. & Rel. Technol.* **2003**, *26(1)*, 29-41.

Anexo IV Sousa, E. P.; Tiritan, M. E.; Oliveira R. V.; Afonso, C. M. M.; Oliveira, R. V.; Cass, Q. B.; Pinto, M. M. M. Enantiomeric Resolution of Kielcorin Derivatives by HPLC on Polysaccharide Stationary Phases Using Multimodal Elution. *Submetido*.

Anexo V Pedro, M. M.; Cerqueira, F.; Sousa, M. E.; Nascimento, M. S. J.; Pinto, M. M. M. Xanthones as Inhibitors of Growth of Human Cancer Cell Lines and Their Effects on the Proliferation of Human Lymphocytes *In Vitro*. *Bioorg. Med. Chem.* **2002**, *10*, 3725-3730.

Anexo VI Saraiva, L.; Fresco, P.; Pinto, E.; Sousa, E.; Pinto, M.; Gonçalves, J. Synthesis and *In Vivo* Modulatory Activity of Protein Kinase C of Xanthone Derivatives. *Bioorg. Med. Chem.* **2002**,*10*, 3219-3227.

Anexo VII Saraiva, L.; Fresco, P.; Pinto, E.; Sousa, E.; Pinto, M.; Gonçalves, J. Inhibition of Protein Kinase C by Synthetic Xanthone Derivatives. *Bioorg. Med. Chem.* **2003**, *11*, 1215-1225.

Anexo VIII Saraiva, L.; Fresco, P.; Pinto, E.; Sousa, E.; Pinto, M.; Gonçalves, J. Inhibitory Effect of Xanthonolignoids on Isoforms α, βI, δ, η, and ζ of Protein Kinase C. *J. Enzyme Inhib. Med. Chem.* **2003**, *18(4)*, 357-370.

As estruturas dos compostos citados ao longo do texto estão localizadas em duas páginas desdobráveis no final da dissertação. A bibliografia é indicada no final de cada Capítulo.

Este projecto centrou-se fundamentalmente em quatro objectivos:

- revisão da química e das actividades biológicas de xantonas e xantonolignóides (***Capítulo 1***),
- planeamento e obtenção de xantonas simples oxigenadas e xantonolignóides, particularmente derivados da série das kielcorinas e isokielcorinas (***Capítulo 2***),
- resolução enantiomérica dos xantonolignóides sintetizados (***Capítulo 3***),
- avaliação da actividade biológica dos compostos sintetizados, estudo de relação estrutura-actividade e avaliação da influência da quiralidade na actividade dos xantonolignóides (***Capítulo 4***).

No Capítulo 1 é apresentada uma revisão das actividades biológicas descritas na literatura para derivados xantónicos. Foram revistas a química e as actividades biológicas para os xantonolignóides (***Anexo I***).

No Capítulo 2 é descrito o planeamento, a síntese e a determinação estrutural de cinco xantonolignóides: *trans*-(±)-kielcorina C (**29**), *cis*-(±)-kielcorina C (**30**), *trans*-(±)-kielcorina D (**31**), *trans*-(±)-isokielcorina D (**32**) e *trans*-(±)-kielcorina E (**33**). A estratégia de síntese baseou-se no acoplamento oxidativo do álcool coniferílico (**36**) com uma *orto*-di-hidroxixantona apropriada. Foi estudada a influência de vários agentes oxidantes no acoplamento oxidativo (***Anexo II***). No sentido de comparar os resultados obtidos em diferentes condições de síntese, foi desenvolvido um método por HPLC-UV de separação e quantificação dos cinco isomeros kielcorínicos (***Anexo III***). Neste capítulo, é também incluída a síntese dos blocos construtores xantónicos e a de xantonas monoxigenadas para ensaios de *screening* e estudos de relação estrutura-actividade (***Anexos II,V- VII***).

Com o objectivo de averiguar a influência da quiralidade na actividade biológica dos xantonolignóides foi desenvolvido um método analítico por HPLC, apresentado no Capítulo 3, para a separação dos enantiómeros kielcorínicos. Estas separações foram analisadas em diferentes fases estacionárias quirais (***Anexo IV***). Quatro misturas racémicas, *trans*-(±)-kielcorina C (**29**), *trans*-(±)-kielcorina D (**31**), *trans*-(±)-isokielcorina D (**32**) e *trans*-(±)-kielcorina E (**33**), foram resolvidas em escala semi-preparativa para a avaliação antitumoral dos respectivos enantiómeros.

Dadas as aplicações da família Clusiaceae na medicina tradicional e pela experiência do grupo de trabalho do CEQOFFUP, pareceu ser de interesse avaliar a actividade antitumoral e imunomoduladora dos compostos referidos. No Capítulo 4 é apresentada a actividade antiproliferativa dos xantonolignóides (***Anexo II***) e das xantonas simples oxigenadas (***Anexo V***) em três linhas celulares tumorais humanas, MCF-7 (adenocarcinoma da mama), TK-10 (carcinoma renal) e UACC-62 (melanoma), e a actividade de enantiómeros kielcorínicos no crescimento da linha celular MCF-7. O estudo da actividade imunomoduladora destes compostos, baseada na inibição da proliferação de linfócitos humanos em resposta à estimulação pela lectina mitogénica fito-hemaglutinina (PHA), é também apresentado (***Anexos II*** e ***V***).

Com a finalidade de procurar novos agentes quimioterápicos baseados na interacção com a PKC, pelos dados de actividades biológicas apresentados no Capítulo 1 para as xantonas simples oxigenadas e dadas as semelhanças estruturais dos xantonolignóides com alguns inibidores da PKC, foram conduzidos estudos *in vitro* e *in vivo* na modulação das isoformas α, βI, δ, η, e ζ, da PKC para estes compostos. Os resultados das xantonas simples oxigenadas (***Anexos VI*** e ***VII***) e dos xantonolignóides sintetizados (***Anexo VIII***) são também discutidos neste Capítulo.

No Capítulo 5 são apresentadas as conclusões desta dissertação. Os resultados obtidos neste trabalho permitiram aprofundar aspectos relacionados com a síntese de derivados da xantona (xanten-9-ona), xantonas simples oxigenadas e xantonolignóides, e estabelecer relações entre a estrutura e as actividades biológicas determinadas. Esta classe de compostos revelou-se interessante no desenvolvimento de antitumorais e imunomoduladores e mostrou ser um importante grupo de activadores e inibidores potentes e selectivos para as várias isoformas da PKC. No entanto, estudos posteriores são necessários para confirmar os mecanismos de acção propostos. Esta dissertação pode contribuir para um futuro planeamento racional de novos compostos farmacologicamente activos, baseados nestes padrões estruturais.

Capítulo 1

Introdução Geral

Nothing in life is to be feared, it is only to be understood.

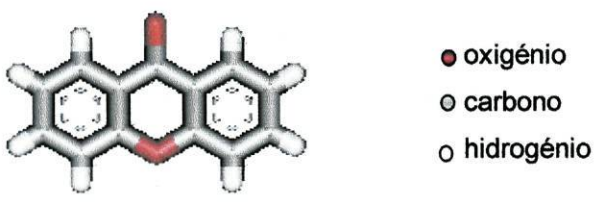
Marie Curie, 1867-1934

A Química Medicinal é uma ciência baseada na Química mas que envolve também aspectos das ciências biológicas, médicas e farmacêuticas. Relaciona-se com a invenção, descoberta, desenho, identificação e preparação de compostos biologicamente activos, com o estudo do seu metabolismo, com a interpretação do seu modo de acção a nível molecular e com o estabelecimento da relação estrutura-actividade [1]. Um dos grandes desafios da Química Medicinal prende-se com a obtenção de estruturas químicas novas que servirão como base para novos agentes terapêuticos. Durante muito tempo, as plantas foram quase exclusivas na terapia disponível para o Homem. Com o desenvolvimento da Química Medicinal no início do século XIX, as plantas representavam ainda o primeiro recurso terapêutico. Actualmente, uma das principais fontes na descoberta de novos fármacos continua a ser baseada na medicina tradicional, com uma fatia significativa a corresponder a produtos naturais, ou a produtos sintéticos baseados em modelos de produtos naturais [2,3].

Algumas plantas contendo xantonas têm sido usadas desde há milhares de anos pela medicina tradicional como é o caso do extracto de *Hypericum perforatum* [4]. Os derivados de origem natural, xantonas e xantonolignóides, têm sido alvo de interesse por parte do CEQOFFUP [5-7] por se tratarem, não só de produtos naturais com reputação em práticas etnomedicinais, como também, por possuírem um número alargado de actividades farmacológicas [8-11]. Desse interesse, resultaram variados estudos que revelaram para xantonas e/ou xantonolignóides actividades como, anti-hepatotóxica [12], inibidora da monoaminoxidase[13], moduladora da quinase C de proteínas (PKC) [14], anticomplementar [15-17] e imunomoduladora [17]. Os estudos realizados não se restringiram à investigação de compostos de origem natural [14,16-18], tendo-se estendido a derivados obtidos por síntese [19-23].

Xantonas

O núcleo da xantona ou xanten-9-ona (dibenzo-γ-pirona, **1**) compreende uma importante secção nos heterociclos oxigenados.



1

De acordo com a natureza dos substituintes presentes na estrutura dibenzo-γ-pirona, as xantonas podem ser divididas em: xantonas simples oxigenadas, xantonas glicosiladas, xantonas preniladas e derivados e xantonas mistas [8,9,24,25]. Alguns autores [9,24,25] incluem nesta classificação os xantonolignóides, compostos que possuem na sua estrutura uma unidade de fenilpropano ligada por uma ponte dioxano ao núcleo xantónico [26].

As xantonas são metabolitos secundários de ocorrência restrita, essencialmente a duas famílias de plantas superiores, Gutifferae (Clusiaceae) e Gentianaceae, e a alguns fungos e líquenes [8-11,24,25,27]. A distribuição uniforme de alguns padrões de oxigenação de xantonas nestas famílias de plantas superiores, despertou o interesse por estes metabolitos para aplicação na classificação quimiossistemática [24,28-33]. Enquanto que a origem biogenética das xantonas em plantas superiores segue a via acetato-xiquimato [34-40], para os fungos e líquenes está proposta a via poliacetato [41-43]. A função das xantonas como metabolitos secundários é ainda pouco clara, tendo sido proposto que poderão servir de mediadores nas interacções entre o organismo que as produz e o ambiente [44]. Podem ser encontradas na literatura algumas revisões interessantes sobre a ocorrência [8-11,24,25,27], a biossíntese [9,24,35], a síntese [34,45] e a determinação estrutural [22,46-49] de xantonas.

O interesse crescente nesta classe de compostos tem sido associado, não só ao seu valor quimiotaxonómico, como mais recentemente, às interessantes propriedades farmacológicas reveladas, quer por produtos de origem natural, quer por derivados obtidos por síntese. As xantonas foram já descritas pelas suas propriedades antioxidantes [50-62], actuando como quelantes de metais [54], na captação de radicais livres [50-52,54,58-60,63-67] e inibindo a peroxidação lipídica [50,52-54,58,62,68]. Estas propriedades foram relacionadas com os efeitos hepatoprotectores [12,69], anti-inflamatórios [62,63] e antidiabéticos [61].

As actividades biológicas desta classe de compostos encontram-se dispersas na literatura. Nas Tabelas 1.1 e 1.2 pretendeu-se fazer uma síntese dos efeitos produzidos pelos derivados xantónicos nos vários sistemas celulares enzimáticos e funcionais. Pela observação destas Tabelas, pode-se concluir que as xantonas apresentam um leque muito alargado de propriedades bioquímicas e farmacológicas. Por essa razão têm sido alvo de muitos estudos por diversos grupos, que têm descrito para esta classe de compostos as actividades que se apresentam na Tabela 1.3.

Tabela 1.1. Efeitos Produzidos por Derivados Xantónicos em Sistemas Celulares Enzimáticos

Sistemas Enzimáticos	**Efeitos**	**Derivados Xantónicos**
Acetilcolinesterase	inibição	aminoalcoxixantonas e seus derivados azoxantónicos [70]
Aldose redutase	inibição	sulfamoilxantona carboxilada [71,72] e xantona carboxilada[73]
Aromatase	inibição	imidazolxantonas e triazolxantonas [74]
Cicloxigenases	inibição	xantonas com epóxidos [75] e xantona prenilada [76]
Citocromo redutase	inibição	extracto de *Mangifera indica* (xantona prenilada como principal constituinte) [69] e ácido 5,6-dimetilxantenon-4-acético (DMXAA, **17**) [77]
Enzima conversora da angiotensina I	inibição	xantonas simples polioxigenadas [78]
Esfingomielinases	inibição	xantonas preniladas [79,80]
Fosfatase do nucleotído monofosfato cíclico de adenosina (AMPc)	inibição não competitiva	xantonas preniladas [81]
3',5'-Fosfodiesterase do AMPc	inibição	xantonas simples oxigenadas [82]
Fosfolipase C	inibição	xantona-cromona [83] e xantona simples tetraoxigenada [59]
17-α-Hidroxilase/C17,20-lipase	inibição	imidazolxantonas e triazolxantonas [74]
11-β-Hidroxilase	inibição	xantona-antraquinona [84]
Hipoxantina-xantina oxidase	inibição	xantona tetraoxigenada [59]
Monoaminoxidases A e B	inibição competitiva, reversível e selectiva	xantona prenilada[85,86], prenilada glicosilada [86], xantona (**1**) [87] e xantonas simples polioxigenadas [13,88-93]
Polimerase do DNA	inibição	xantona-flavona glicosilada [94]
Proteases:	inibição não	
HIV-1	competitiva	xantona-flavona glicosilada [95]
	inibição	xantonas preniladas [95,96]
Aspártica	inibição	xantonas simples polioxigenadas e preniladas [97]
Caspase-3	activação	xantonas preniladas [98]
Quinases:		
Fosfoquinase da creatinina	inibição	xantona glicosilada [61]
Quinase A de proteínas (PKA)	inibição	xantonas preniladas [81,99]
Quinase C de proteínas (PKC)	inibição	xantonas preniladas [81,99] e xantona simples tetraoxigenada [55]
	activação	1,7-di-hidroxixantona (**16**) [14,100]
Quinase de proteínas dependente de cálcio (CDPK)	inibição	xantonas preniladas [81]
Quinase IkappaB (IKK)	inibição	DMXAA (**17**) [101]
Quinase cadeia leve da miosina (MLCK)	inibição	xantonas preniladas [81]
Quinona oxidorredutase de (fosfato de) nucleótido de nicotinamida e adenina (NAD(P)H)	inibição	DMXAA (**17**) [77]
Sulfotransferases	inibição	xantonas tetraoxigenadas [102]
	substrato	xantonas poli-hidroxiladas [103]
Topoisomerases I e II	inibição	furanoxantona psorospermina (**18**) [104,105], xantonas simples oxigenadas, preniladas, benzopiranoxantonas, furanoxantonas e um xantonolignóide [106]
Transcriptases reversas:		
vírus de leucemia murina de Moloney (MMLV)	inibição	xantonas simples oxigenadas [107]
vírus da imunodeficiência do tipo 1 (HIV-1)	inibição	xantona-flavona glicosilada [94,108] e benzopiranoxantonas [109]

Tabela 1.2. Efeitos Produzidos por Derivados Xantónicos em Sistemas Celulares Funcionais

Sistemas funcionais	**Efeitos**	**Derivados xantónicos**
Albumina	ligação	xantonas com grupos carboxilo e tetrazolilo [110]
Canais de cálcio	bloqueio	xantona [111,112], xantona tetraoxigenada [112], xantonas com grupos metanofosfónico [113], com aneis piridínicos [114-120] e com cadeias propanolamina [121]
Glicoproteínas:		
P	modulação	xantonas preniladas [122]
CD4 (*cluster of differentiation 4*)	ligação	xantonas policíclicas [123]
Hemoglobina	formação de um complexo com o dímero do grupo heme	xantona dioxigenada [124] e várias nitroxantonas [125]
	redução da afinidade para o oxigénio	xantona com um grupo tetrazolilo [126]
Linfócitos T	inibição da proliferação	xantonas polioxigenadas [17]
	activação/inibição da proliferação	xantona glicosilada [127]
Macrófagos	inibição da actividade fagocítica	extracto de *Mangifera indica* [65] e xantona glicosilada [65,128]
	inibição da produção de espécies reactivas de oxigénio e	extracto de *Mangifera indica* [65], xantona glicosilada [65] e 4-hidroxixantona (**9**) [129]
	inibição da produção de óxido nitroso	
	estimulação da produção de óxido nitroso	DMXAA (**17**) [130]
Proteína HSP-47	inibição da expressão genética	xantona policíclica [131]
Receptores β-adrenérgicos	bloqueio	xantonas com cadeias propanolaminas [121,132,133]
Receptores 2^A da 5-hidroxitriptamina	bloqueio	DMXAA (**17**) [134] e xantona prenilada [135]
Receptores do factor de activação plaquetária	antagonistas	xantonas preniladas [136,137]
Receptores do leucotrieno B4	bloqueio	xantonas carboxiladas [138-140] e com substituintes bifenilo [141]
Receptores da prostaglandina E2	bloqueio	xantona prenilada [76] e carboxilada [138]
Sistema humano do complemento	inibição	xantonas monoxigenadas [15], polioxigenadas [16,17] e preniladas [17]

Tabela 1.3. Actividades Biológicas Descritas para a Classe das Xantonas

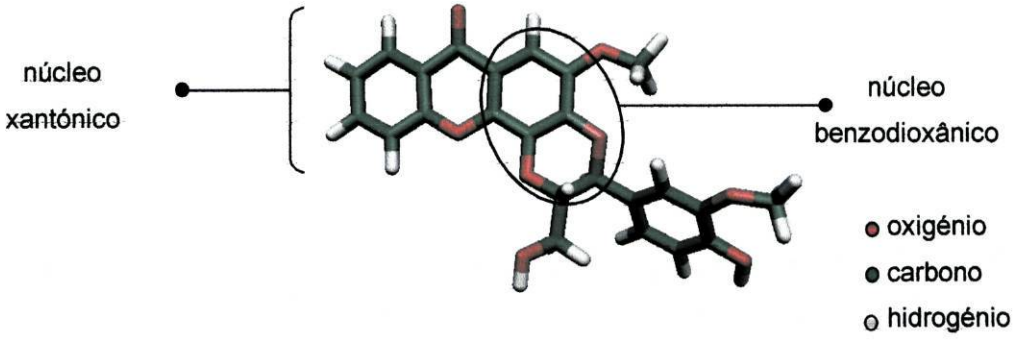
Actividades Biológicas	**Alvos / Mediadores / Processos Associados**
Anti-infeciosos:	
Antibacterianos [97,142-153]:	
Tuberculostáticos [148,154-159]	
Antifúngicos [81,90,97,142,143,160-172]	protease aspártica secretada [97], PKA [81*,99]
Antivíricos [173-176]:	replicação [173,174]
Antirretrovíricos HIV-1 [94-96,107-109,128,177-179]	transcriptase reversa do HIV-1 [94*,108*,109*,177], transcriptase reversa do MMLV [107], protease do HIV-1 [95*,96*], glicoproteína CD4 [123*]
Antimaláricos [3,124,180-187]	heme [126,182,186], CDPK [81*]
Depressores do SNC [154,188-190]	
Estimulantes do SNC [191-193]	monoaminoxigenases [85*]
Anticonvulcionantes/ Antiepiléticos [194-199]	
Analgésicos [200,201]	bradicinina, substância P [201]
Antiarrítmicos [199,202-204]	canais de cálcio [114-117], peroxidação lipídica [204]
Anti-hipertensores / Vasodilatadores [111,121,134,202,205,206]	canais de cálcio [111-116*,121], AMPc [111], 5-hidroxitriptamina [134], receptores β-adrenérgicos [121,133*], mastócitos (desgranulação) [205], factor de activação plaquetária [206], enzima conversora da angiotensina [78*]
Diuréticos [207]	
Anti-hiperlipidémicos [208]	
Antiplaquetários / Anticoagulantes [75,136,206,209-214]	cicloxigenases [75], factor de activação plaquetária [136,206], tromboxano [209,210], fosfato de inositol [209]
Antiasmáticos [215-217]	mastócitos (desgranulação) [217,218*], acetilcolina, histamina, bradicinina, substância P, prostaglandina E2, tromboxano A2 [216], receptores do leucotrieno B4 [138-141*]
Anti-hepatotóxicos [12,69]	citocromo P-450 redutase dependente do NADPH [69]
Antidiabéticos [61,71-73,219-226]	aldose redutase [71-73], fosfoquinase da creatinina [61], insulina [220,223], glucose [218,222,223,225], colesterol [222,224], triglicerídeos [224,225], hemoglobina glicosilada [61]
Antiosteoporóticos [227]	
Anti-inflamatórios [63,76,190,200,216,228,229]	receptores das prostaglandinas E2 e cicloxigenases [76], beta-glucuronidase, lisozima, histamina, mastócitos e neutrófilos (desgranulação) [63], imunoglobulina E [231], acetilcolina, histamina, bradicinina, substância P, prostaglandina E2 e tromboxano A2 [216], PKC [55*,81*,99*,100*], fosfolipase C [59*,83*]
Antialérgicos [216,218,230-234]	mastócitos (desgranulação) [218,229], acetilcolina, histamina, bradicinina, substância P, prostaglandina E2, tromboxano A2 [216], imunoglobulina E [230], receptores do leucotrieno B4 [138-141*]
Antianafiláticos [200,205,229-231,235]	imunoglobulina E [229,230,235], imunoglobulina G [235], mastócitos (desgranulação) [205]
Antineoplásicos e Quimiopreventivos	ver Capítulo 4
Antiulcerosos [191,236]	*Helicobacter pylori* [236]

* alvos directamente avaliados sem investigação da actividade referida.

Os resultados promissores da avaliação das actividade farmacológicas deram origem a um crescente interesse na optimização dos compostos líderes ou dos "hits" revelados. Actualmente, encontram-se disponíveis vários estudos de relação estrutura-actividade para as xantonas [57,62-64,70,73,74,81,82,87,99,116,118-120,122,133,139,141,142,155,157,159,168,176,182,185,208-211,213,214,228,230,231,233,234,237-246].

Xantonolignóides

Na estrutura dos xantonolignóides sobressaem duas unidades fundamentais com potencial actividade biológica, como se exemplifica para a *trans*-(±)-kielcorina (**27**): o núcleo xantónico e o núcleo 1,4-benzodioxânico.



27

Compostos com o núcleo 1,4-benzodioxânico têm sido amplamente estudados pelas suas propriedades anti-hipertensivas como potentes antagonistas α-adrenérgicos [247-258] e com outras acções a nível do sistema nervoso central [259-261]. Este núcleo está presente numa variedade de lignanos [27,262-268], nos quais se destaca a silibina (**39**), um flavonolignóide cuja actividade anti-hepatotóxica [269,270] foi já relacionada às suas propriedades antioxidantes [263,271], actuando como quelante de metais [272] e inibidor da peroxidação lipídica [273]. Considerando as semelhanças estruturais e electrónicas dos xantonolignóides com a silibina (**39**), foram já avaliadas a actividade anti-hepatotóxica da *trans*-(±)-kielcorina (**27**) e da *trans*-(±)-isokielcorina B (**35**) [12]. Recentemente, a silibina (**39**) surgiu como moduladora da glicoproteína P [274], permitindo a sensibilização de espécies de *Leishmania* resistentes à daunomicina [275].

A presença das unidades estruturais xantona/benzodioxano no esqueleto dos xantonolignóides, torna-os uma classe de moléculas promissoras para estudos de actividades biológicas. O ***Anexo I*** (pág. 95-107) descreve a ocorrência, o isolamento, a determinação

estrutural, a síntese a as actividades biológicas de xantonolignóides. A variabilidade estereoquímica conferida pela presença de dois centros estereogénicos e a forma global que estas molélulas adquirem pela variação da posição da ponte dioxano, relativamente ao núcleo xantónico, constituem desafios interessantes no desenvolvimento de estudos de relação estrutura-actividade e na procura de novos representantes desta classe de compostos.

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Capítulo 2

Síntese e Elucidação Estrutural de Xantonas e Análogos da Kielcorina

One of our strongest capabilities is in synthetic chemistry, which is basically the ability to make virtually anything, any compound we want. This allows us to explore areas that other research groups might hesitate to consider because they are too difficult, to involved, too complicated.

Robert Young, Vice-Presidente da Merck Frosst, Química Medicinal [1]

A síntese de xantonas introduzida por Grover, Shah e Shah em 1955 [2], envolvendo reagentes acessíveis e uma metodologia simples, goza ainda de grande popularidade. Requer um derivado do ácido salicílico e um fenol apropriado sendo estes compostos submetidos a aquecimento, na presença de um agente desidratante como o cloreto de zinco, em pentacloreto de fósforo. Devido a limitações apresentadas por este processo, outras metodologias têm sido utilizadas na síntese de xantonas simples oxigenadas [3] (***Anexo V***, pág. 161). Destas destacam-se:

- a síntese de Ullmann, através de intermediários de éter bifenílico contendo um grupo carboxilo, que cicliza por uma acilação intramolecular ácido catalizada [4];
- a formação de intermediários do tipo benzofenona introduzida por Scheinmann, por acilação de Friedel-Crafts de derivados metoxibenzeno com cloretos de benzoílo apropriados na presença de cloreto de alumínio em éter; a ciclização a xantonas efectua-se pela eliminação base-catalizada de metanol [5].

Os xantonolignóides têm sido sintetizados por dois processos distintos (***Anexo I***, pág. 102-104): pela via clássica [6,7], e pela via biomimética [8-10]. A primeira, a síntese clássica, baseia-se na escolha de sintons e dos respectivos equivalentes sintéticos [7]. A segunda, a síntese biomimética, é planeada a partir dos blocos construtores usados *in vivo* [11]. Pinto *et al.*[12] compararam, para a *trans*-(±)-kielcorina (**27**), as duas estratégias de síntese, clássica *versus* biomimética. A obtenção de xantonolignóides pela via clássica exigiu um elevado número de passos em condições reaccionais consideradas drásticas, o que contrastou substancialmente com a via biomimética, que possibilitou uma redução do número de etapas e a utilização de condições suaves de reacção, nomeadamente de pH e temperatura [11,12]. O caminho biogenético proposto para a *trans*-(±)-kielcorina (**27**) foi mimetizado usando como blocos precursores a 3,4-di-hidroxi-2-metoxixantona (**19**) e o álcool coniferílico (**36**) e foi obtido igualmente o estereoisómero *cis*-(±)-kielcorina (**28**) [8]. Mais tarde, no acoplamento biomimético da 2,3-di-hidroxi-4-metoxixantona (**20**) com o álcool coniferílico (**36**) foi obtida a *trans*-(±)-kielcorina B (**34**) e o seu regioisómero, a *trans*-(±)-isokielcorina B (**35**) [10].

Planeamento

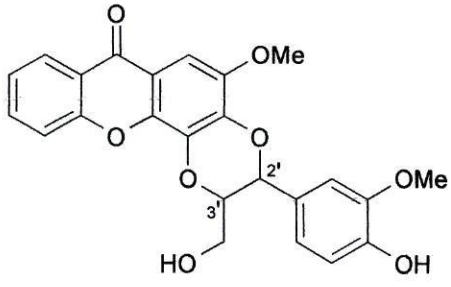
Ao planear os análogos kielcorínicos a obter, foi considerado como modelo a molécula *trans*-(±)-kielcorina (**27**), que pode ser constituída por dois sintons (**I**) e (**II**):



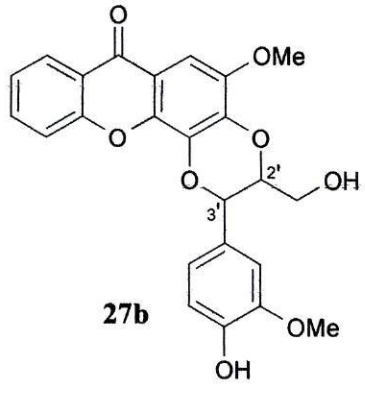
27

correspondendo os equivalentes sintéticos à 3,4-di-hidroxi-2-metoxixantona (**19**) e ao álcool coniferílico (**36**), respectivamente.

No processo de acoplamento oxidativo, ambos os hidroxilos fenólicos da 3,4-di-hidroxi-2-metoxixantona (**19**) podem sofrer ataque pelo álcool coniferílico (**36**) e originar dois regioisómeros: a kielcorina (**27a**), com o padrão de substituição 3'-hidroximetil-2'-aril-, e a isokielcorina, com o padrão de substituição 2'-hidroximetil-3'-aril- (**27b**).

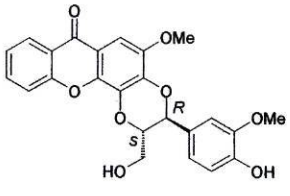


27a

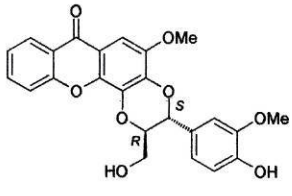


27b

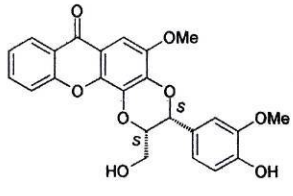
Cada um dos regioisómeros, **27a** e **27b**, possui dois centros estereogénicos podendo assim representar quatro estereoisómeros. Exemplificando, a kielcorina **27a** pode corresponder a um dos quatro enantiómeros: a *(S,R)*-kielcorina (**27a I**), a *(R,S)*-kielcorina (**27a II**), a *(S,S)*-kielcorina (**27a III**) e a *(R,R)*-kielcorina (**27a IV**).



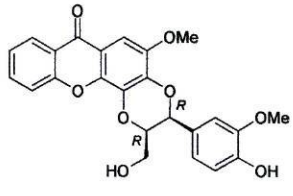
27a I



27a II



27a III



27a IV

Como consequência, do acoplamento de uma *orto*-di-hidroxixantona com uma unidade fenilpropano pode prever-se a formação de oito compostos. Adicionalmente, observando os enantiómeros **I-IV**, verifica-se que os substituintes hidroximetilo e arilo no anel dioxano podem encontrar-se em planos opostos e corresponder a *trans* diasterioisómeros (**I/II**) ou no mesmo plano e corresponder a *cis* diasterioisómeros (**III/IV**).

Tendo em consideração a definição dos xantonolignóides - compostos que possuem na sua estrutura uma unidade de fenilpropano ligada, por uma ponte dioxano, a um núcleo xantónico – existem três possibilidades de uma *orto*-di-hidroxissubstituição, isto é, a ponte dioxano pode localizar-se na aresta (posição) *a* (1,2-), *b* (2,3-) ou *c* (3,4-) do núcleo xantónico. Assim, explorando a posição da porção C(6)-C(3), podem ser definidas três formas globais que se designam por: "long-chair", "linear" e "pistola" (Fig. 2.1).

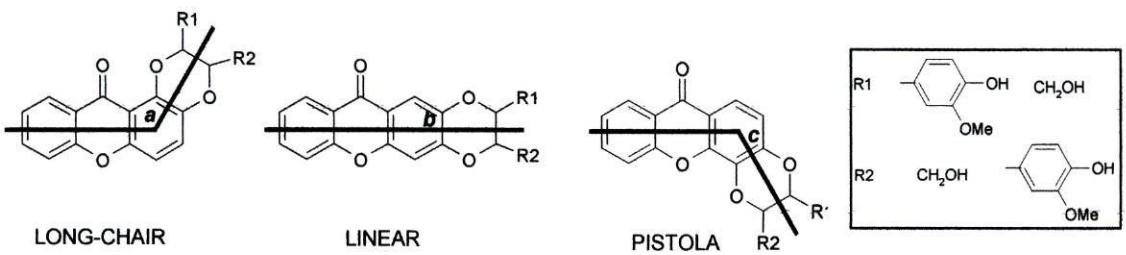


Fig 2.1. Formas globais para as estruturas kielcorínicas.

Tendo como modelo o procedimento anteriormente descrito [8] para a síntese biomimética da *trans*-(±)-kielcorina (**27**) (***Anexo I***, Fig. (7)), a obtenção das três formas globais de isómeros constitucionais foi baseada no acoplamento oxidativo do álcool coniferílico (**36**) com uma *orto*-di-hidroxixantona: a 1,2-di-hidroxixantona (**11**), a 2,3-di-hidroxixantona (**13**) e a 3,4-di-hidroxixantona (**15**), blocos construtores para os derivados kielcorínicos "long-chair", "linear" e "pistola", respectivamente (Fig. 2.2).

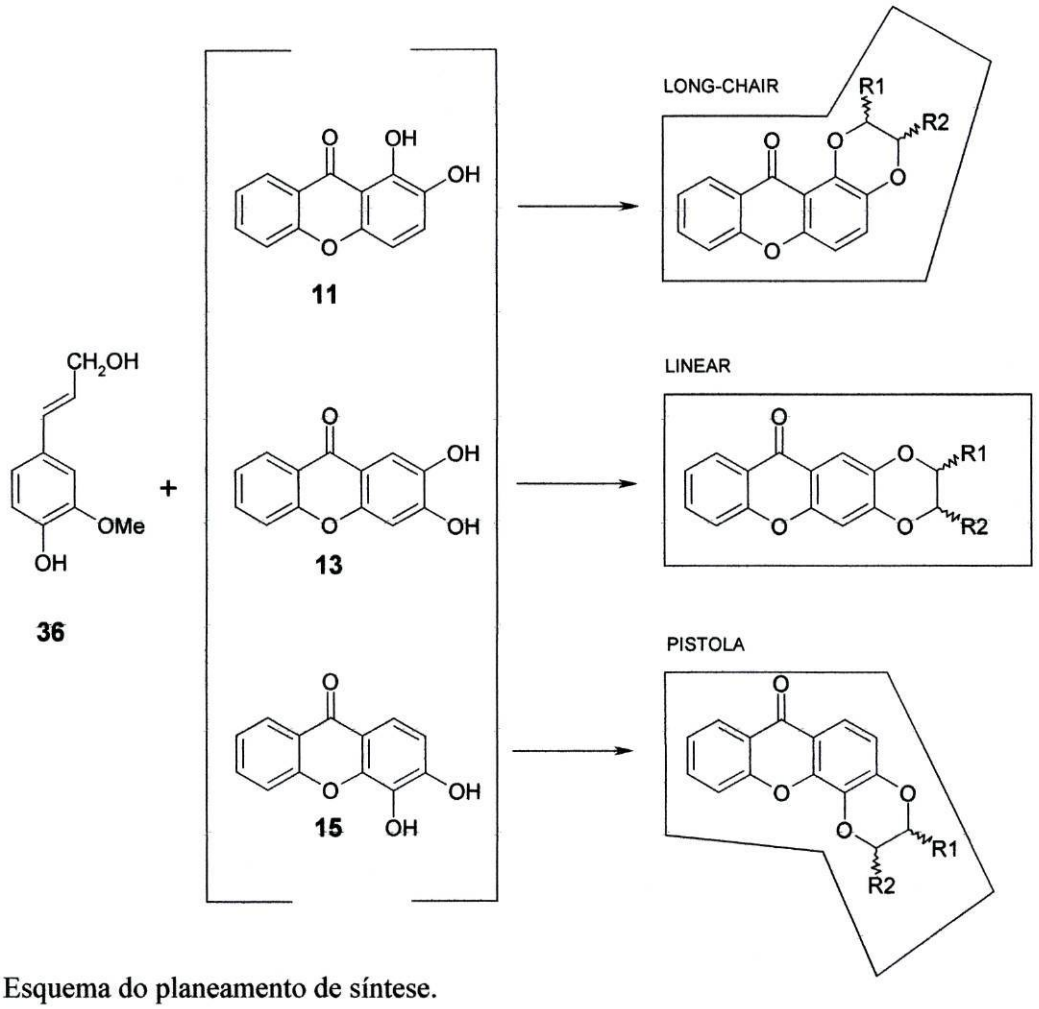


Fig. 2.2. Esquema do planeamento de síntese.

O planeamento apresentado inclui também uma modificação molecular em relação à kielcorina **27** que se reflecte na ausência do substituinte metoxilo adjacente ao grupo catecol do núcleo xantónico. Esta modificação permite, não só determinar a importância desta depleção na actividade biológica, como também simplificar a síntese dos análogos kielcorínicos.

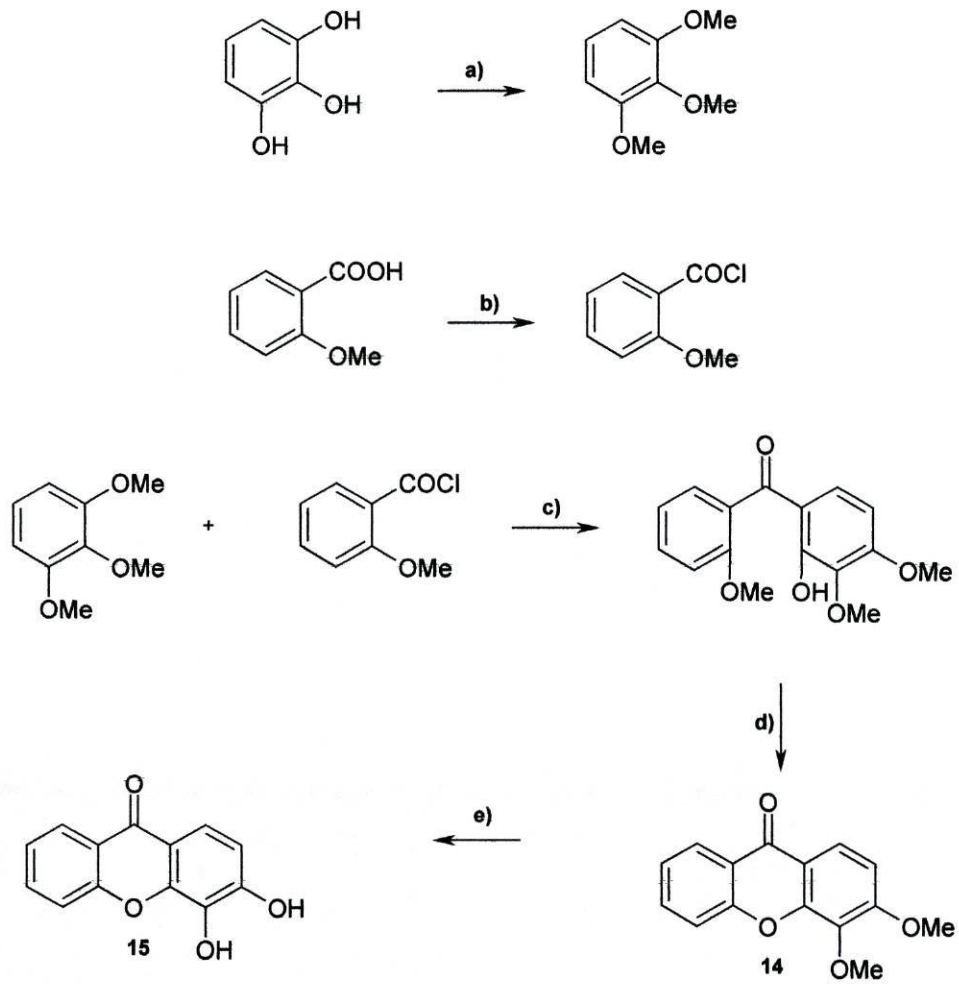
Procedimento Geral de Síntese

Síntese dos Derivados Xantónicos

A síntese dos blocos construtores xantónicos, 1,2- (**11**) e 2,3-di-hidroxixantona (**13**) e dos intermediários **10**, **12**, **25-26** encontra-se descrita no ***Anexo II*** (pág. 108-123). No sentido de estabelecer estudos de relação estrutura-actividade em xantonas simples oxigenadas, foram

sintetizadas a 1-metoxi- (**2**), a 1-hidroxi- (**3**), a 2-metoxi- (**4**), a 2-hidroxi- (**5**), a 3-metoxi- (**6**) e a 3-hidroxixantona (**7**). O ***Anexo V*** (pág. 159-165) descreve a síntese destes derivados monoxigenados e integra a síntese da 3,4-dimetoxi- (**14**) e 3,4-di-hidroxixantona (**15**), bem como a de outros derivados xantónicos obtidos no CEQOFFUP.

A formação de benzofenonas como intermediários ciclizáveis foi o método utilizado para a obtenção das seguintes xantonas oxigenadas: a 3,4-dimetoxi- (**14**), a 3,4-di-hidroxi- (**15**), a 2-metoxi- (**4**), a 2-hidroxi- (**5**), a 3-metoxi- (**6**) e a 3-hidroxixantona (**7**). Na Fig. 2.3 exemplifica-se para a 3,4-dimetoxi- (**14**) e 3,4-di-hidroxixantona (**15**) a estratégia geral seguida para a síntese de xantonas com estes padrões de substituição.



a) Me_2CO, K_2CO_3, Me_2SO_4, refluxo/24h
b) $SOCl_2$, $C_6H_5CH_3$, refluxo
c) Me_2O, $AlCl_3$, temp.amb./22h
d) MeOH, H_2O, NaOH, refluxo/47h
e) C_6H_6, $AlCl_3$, 70ºC/13h

Fig. 2.3. Via reaccional benzofenona para a obtenção das xantonas dioxigenadas **14** e **15**.

Pela reacção de Nencki [13], derivada do método de Grover, Shah e Shah [2], obteve-se a 1-hidroxi- (**3**) e a 1-metoxixantona (**2**); o caminho sintético e as condições reaccionais são apresentados na Fig. 2.4.

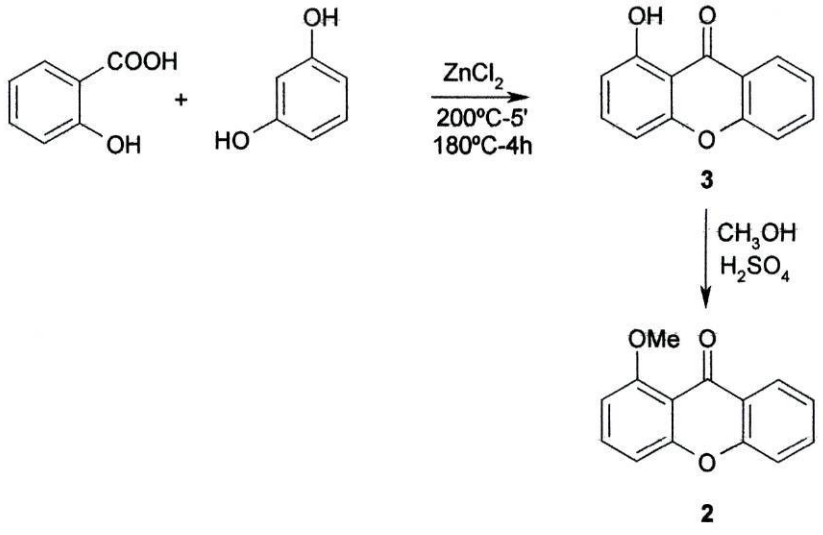


Fig. 2.4. Síntese das xantonas monoxigenadas **2** e **3** pela reacção de Nencki.

As xantonas 1,2- e 2,3-dioxigenadas **10-13** foram obtidas pela via reaccional de Ullmann (Fig. 2.5). Observando a via clássica através da formação de benzofenonas (Fig. 2.3, **c-d**)) e de éteres bifenílicos (Fig. 2.5, **a-c**)), verifica-se que o padrão de substituição para a síntese da 1,2-di-hidroxixantona (**11**) leva, segundo as regras de substituição aromática electrofílica [5], à obtenção das xantonas 2,3-dioxigenadas **12** e **13** e não dos isómeros posicionais **10** e **11**. Daí que, os dados anteriormente descritos para a síntese da 1,2-di-hidroxixantona (**11**) [14] seguiam um processo de síntese de Ullmann para obter inicialmente a 2-hidroxixantona (**5**) com uma posterior formilação de Duff para obter a 1-formil-2-hidroxixantona e finalmente uma oxidação de Dakin para obter o composto **11**. No entanto, foi recentemente sintetizado o composto natural 2-hidroxi-1-metoxixantona (**21**) por uma via regioespecífica ditada por efeitos de coordenação, que consistiu na ciclização de éteres bifenílicos protegidos e na utilização de diisopropilamida de lítio (LDA) [15]. A adaptação deste processo, com a litiação directa [16] do ácido 2-(3,4-dimetoxifenoxi)-benzóico (**26**) desprotegido, e do processo descrito por Jackson *et al.* [17] para a reacção de Ullmann [4,14] permitiram a optimização do processo de síntese para a 1,2-di-hidroxixantona (**11**), apresentado no ***Anexo II***.

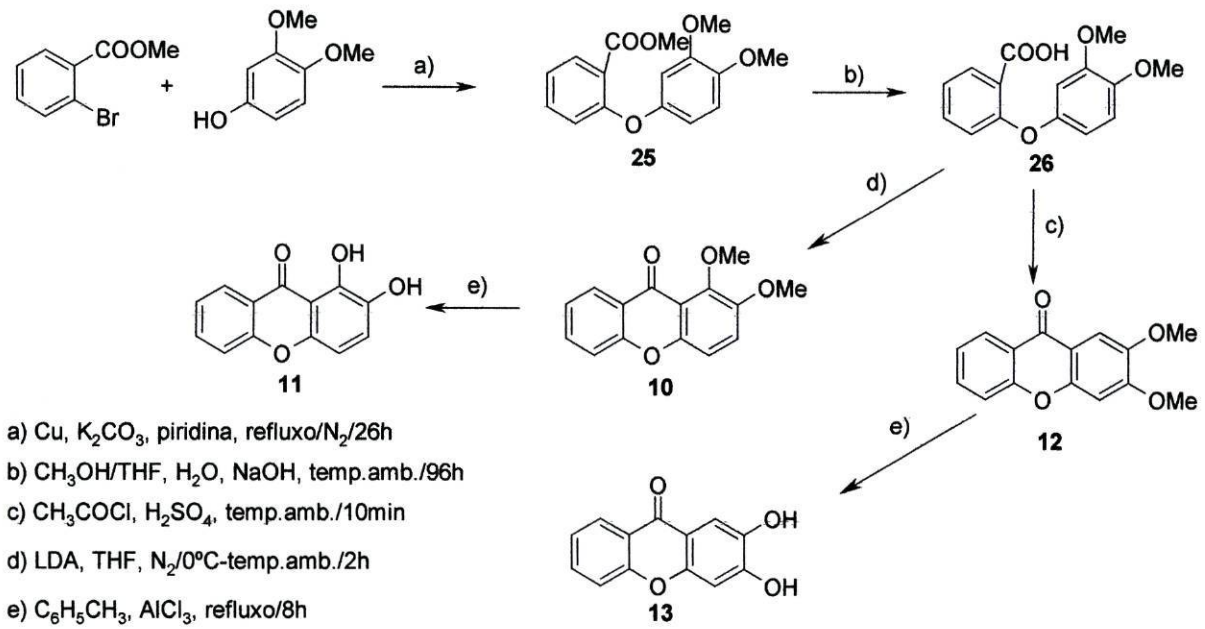


Fig. 2.5. Via reaccional de Ullmann para a obtenção das xantonas dioxigenadas **10-13**.

Quando comparada com a síntese via benzofenona de xantonas 2,3-dioxigenadas [5], a reacção de Ullmann clássica origina rendimentos baixos [18,19]. No entanto, para a síntese da 2,3-di-hidroxixantona (**13**) optou-se pela via de Ullmann com a adaptação descrita por Jackson *et al.* [17] (Fig. 2.5, **a)-b)**), na medida em que esta melhorou consideravelmente o rendimento da síntese do éter bifenílico **25**.

Síntese de Análogos da Kielcorina

No ***Anexo II*** (pág. 108-123) é descrita a síntese dos isómeros kielcorínicos planeados. Inicialmente, na reacção de acoplamento oxidativo foi utilizado o óxido de prata como o agente oxidante. A escolha deste agente baseou-se em resultados anteriores: para sintetizar o xantonolignóide natural kielcorina (**27**) foi obtido com o óxido de prata apenas o regioisómero 3'-hidroximetil-2'-aril- [8], enquanto que na síntese do composto natural kielcorina B (**34**) com o carbonato de prata foram obtidos os dois regioisómeros possíveis **34** e **35** [10].

Com o objectivo de obter a *trans*-(±)-kielcorina C (**29**) procedeu-se ao acoplamento oxidativo da 3,4-di-hidroxixantona (**15**) com o álcool coniferílico (**36**) usando como agente oxidante o óxido de prata [8,9,20]. O produto bruto resultante do acoplamento oxidativo foi purificado por cromatografia em coluna. Algumas das fracções obtidas foram purificadas por

cromatografia em camada fina, nas quais se identificou a *trans*-(±)-kielcorina C (**29**) e a *cis*-(±)-kielcorina C (**30**); o isómero *trans* foi obtido em maior quantidade.

Com o objectivo de obter a *trans*-(±)-kielcorina D (**31**) procedeu-se ao acoplamento oxidativo da 1,2-di-hidroxixantona (**11**) com o álcool coniferílico (**36**) usando como agente oxidante o óxido de prata [8,9,20]. Após purificação, identificou-se a *trans*-(±)-kielcorina D (**31**) e, em menor quantidade, o regioisómero *trans*-(±)-isokielcorina D (**32**).

Com a finalidade de obter a *trans*-(±)-kielcorina E (**33**) procedeu-se ao acoplamento oxidativo da 2,3-di-hidroxixantona (**13**) com o álcool coniferílico (**36**) usando como agente oxidante o óxido de prata [8,9,20]. O produto bruto resultante do acoplamento oxidativo foi purificado por cromatografia em coluna tendo-se identificado a *trans*-(±)-kielcorina E (**33**). Nem o regioisómero *trans*-(±)-isokielcorina E, nem os isómeros *cis* foram encontrados como produtos reaccionais.

Em todas as reacções, foram também identificados o álcool desidrodiconiferílico (**37**) e o fenilcumarano (**38**), dois compostos resultantes do acoplamento oxidativo entre duas moléculas de álcool coniferílico (**36**).

Estudos no Processo de Síntese dos Xantonolignóides

Estereoquímica

A obtenção dos estereoisómeros *trans*-(±)-kielcorina C (**29**) e *cis*-(±)-kielcorina C (**30**) em mistura, exigiu o isolamento destes compostos, o qual foi efectuado por cromatografia preparativa em placa. Com o intuito de obter o composto biologicamente mais activo (***Anexo II***, *Table 2*), foi posteriormente conseguida a conversão do estereoisómero *cis* em *trans*. O procedimento apresentado no ***Anexo II*** (pág. 108-123) melhorou expressivamente o processo de síntese de xantonolignóides com a configuração *trans*.

Regioquímica

Somente para a forma global "long-chair" a regioselectividade do acoplamento oxidativo não foi conseguida, utilizando o óxido de prata como agente oxidante. Para justificar este resultado, foram colocadas as seguintes hipóteses:

1. a regioselectividade da reacção não dependeria do agente oxidante utilizado nesta classe de compostos,
2. a regioselectividade da reacção dependeria dos blocos construtores xantónicos.

Estudos em 1,4-benzodioxanos revelaram elevada regiosselectividade com a utilização de sais de prata (óxido e carbonato) na síntese de regioisómeros 3-aril-1,4-benzodioxanos, e pela utilização de hexacianoferrato de potássio na síntese de regioisómeros 2-aril-1,4-benzodioxanos [20]. Baseados nestes resultados, e no sentido de confirmar a primeira hipótese postulada, é descrito no ***Anexo II*** (pág. 108-123) o estudo comparativo de estratégias de síntese no acoplamento oxidativo, com os mesmos agentes oxidantes que os utilizados por Wagner *et col.* [20]. A interferência dos vários agentes oxidantes na natureza e quantidade dos isómeros kielcorínicos obtidos foi avaliada por HPLC. O ***Anexo III*** (pág. 124-137) revela o estudo decorrido da separação dos isómeros kielcorínicos e da sua quantificação, no produto bruto da reacção, utilizando uma metodologia de HPLC-UV.

Para analisar se a regioselectividade da reacção dependeria dos blocos construtores xantónicos foi avaliada a influência da distribuição da densidade electrónica no precursor xantónico, para cada uma das séries (Fig. 2.6-2.8). O estudo do efeito da posição dos grupos substituintes na formação de diferentes regioisómeros foi baseado no mecanismo de reacção radicalar, proposto para a síntese biomimética da kielcorina (**27**) (***Anexo I***, Fig. (5)).

De acordo com a teoria orbital de Frontier, o ponto preferencial de formação do radical fenóxido pode ser previsto pelo efeito da posição dos grupos substituintes no núcleo catecol; dependendo da propriedade retiradora ou dadora de electrões, este núcleo pode conferir, respectivamente, um maior carácter electrofílico ou nucleofílico ao radical [20]. Em estudos com 1,4-benzodioxanos [21] foi demonstrado que o substituinte no anel benzénico que suporta o catecol tem uma influência determinante na regiosselectividade da reacção de acoplamento oxidativo; esta reacção mostrou-se altamente selectiva quando o núcleo catecol possuía substituintes dadores de electrões e muito menos regiosselectiva com catecóis contendo substituintes retiradores de electrões. Deste modo, o bloco construtor xantónico contendo grupos retiradores de densidade electrónica poderá dificultar a oxidação de um hidroxilo e assim induzir menor regiosselectividade ao tornar similar o potencial dos dois hidroxilos, originando como consequência a misturas de regioisómeros.

Considerando a 3,4-di-hidroxixantona (**15**) (Fig. 2.6):

- a densidade electrónica do hidroxilo em C(3) é determinada pelo efeito retirador de electrões do carbonilo em *para*;

- a densidade electrónica do hidroxilo em C(4) é determinada pelo efeito indutivo (-I) do heteroátomo da posição vizinha;

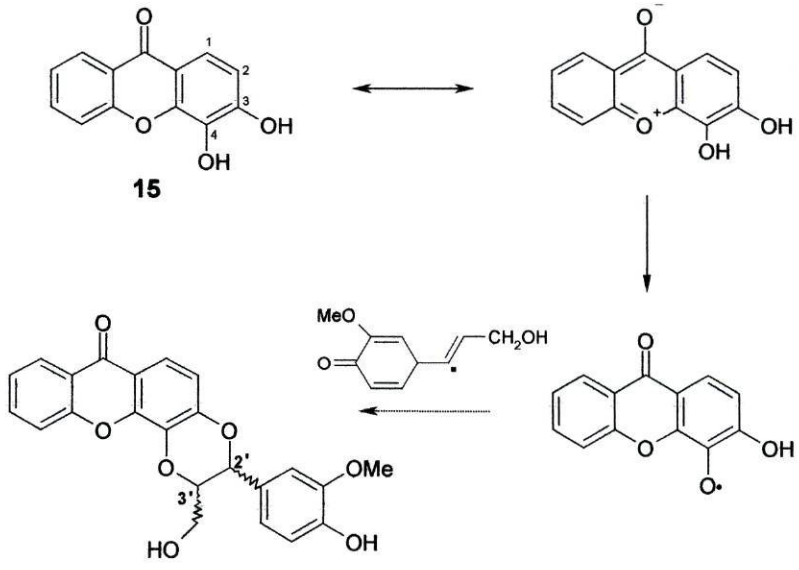


Fig. 2.6. Mecanismo proposto para a formação do regioisómero "pistola" predominante.

- considerando o efeito mesomérico como mais forte que o efeito indutivo, esperar-se-ia existir maior densidade electrónica a nível do hidroxilo em C(4), o que favorecia a sua oxidação; poder-se-ia prever que o radical livre correspondente (Fig. 2.6) seria o que se formaria em maior quantidade no meio reaccional e, consequentemente, obter-se-ia maior quantidade do derivado 3'-hidroximetil-2'-aril-kielcorínico, hipótese que foi confirmada experimentalmente.

Considerando a 1,2-di-hidroxixantona (**11**) (Fig. 2.7):

- a densidade electrónica do hidroxilo em C(1) é determinada pelo efeito retirador de electrões do carbonilo em posição *peri-* [22]; de notar que quando comparada com a posição C(3) na 3,4- (**15**) e 2,3-di-hidroxixantonas (**13**), esta densidade pode encontrar-se mais comprometida, devido a possíveis quelatações com o reagente oxidante;
- no hidroxilo em C(2) não é possível a conjugação mesomérica com o carbonilo e o oxigénio do heterocíclo está demasiado afastado para exercer o seu efeito indutivo (-I) [22];

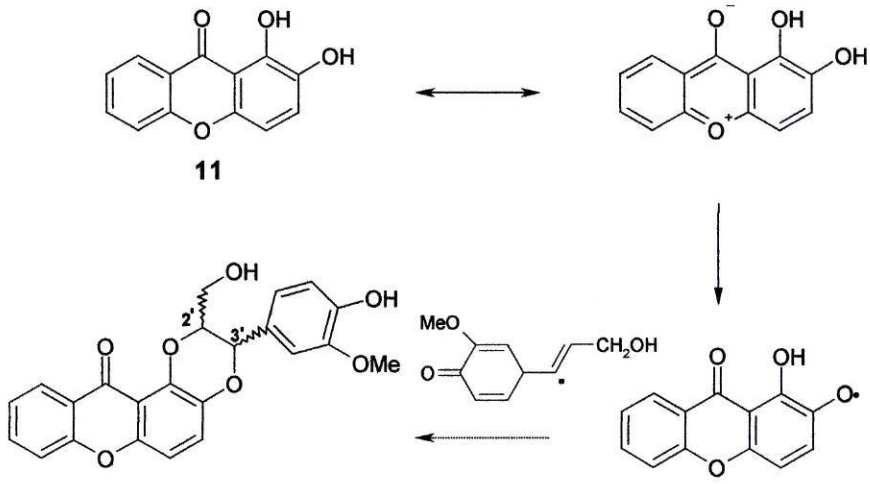


Fig. 2.7. Mecanismo proposto para a formação do regioisómero "long-chair" predominante.

- a possibilidade de esta reacção ser assistida pela formação de um complexo de prata com a ligação dupla da cadeia lateral do álcool coniferilíco (**36**) poderá ser considerada, não havendo, até à data, qualquer estudo nesse sentido. Na ausência de dados que permitissem a quantificação de uma possível quelatação com o reagente oxidante, e apenas considerando o efeito mesomérico, esperar-se-ia existir maior densidade electrónica a nível do hidroxilo em C(2), o que favorecia a sua oxidação; poder-se-ia prever a formação do radical livre correspondente (Fig. 2.7) como o predominante no meio reaccional e, consequentemente, obter-se-ia maior quantidade do derivado 2'-hidroximetil-3'-aril-kielcorínico, hipótese que se verificou experimentalmente.

Considerando a 2,3-di-hidroxixantona (**13**) (Fig. 2.8):

- a densidade electrónica do hidroxilo em C(3) é determinada pelo efeito retirador de electrões do carbonilo em *para*;
- no hidroxilo em C(2) não é possível a conjugação mesomérica com o carbonilo e o oxigénio do heterociclo encontra-se demasiado afastado para exercer o seu efeito indutivo (-I) [22];

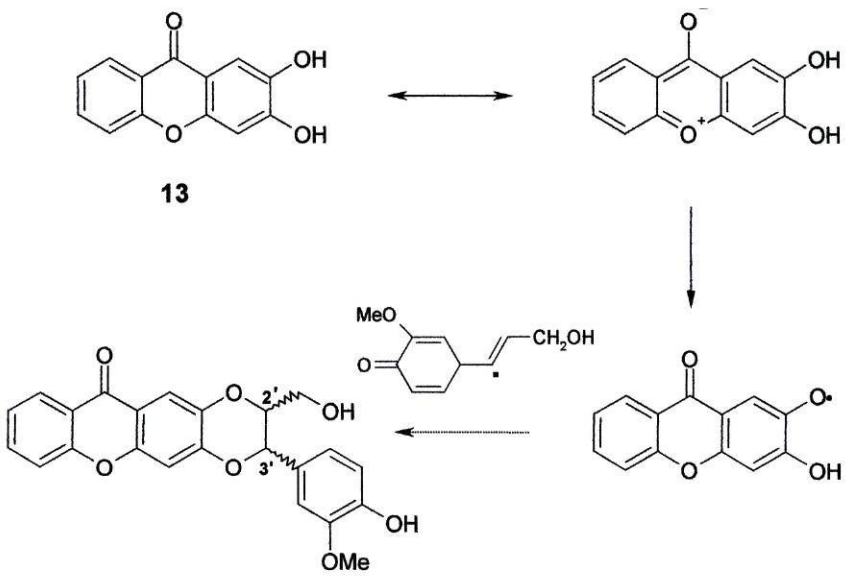
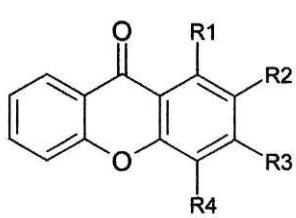


Fig. 2.8. Mecanismo proposto para a formação do regioisómero

- esperar-se-ia existir maior densidade electrónica a nível do hidroxilo em C(2) do que em C(3), o que favorecia a sua oxidação; poder-se-ia prever que o radical livre correspondente (Fig. 2.8) seria o que se formaria em maior quantidade no meio reaccional e, consequentemente, obter-se-ia maior quantidade do derivado 2'-hidroximetil-3'-aril-kielcorínico, hipótese que foi verificada experimentalmente.

Para confirmar esta análise, e consequentemente a segunda hipótese postulada, realizou-se um estudo computacional sobre a estabilidade dos radicais fenóxido possíveis **I-VI** para os blocos construtores xantónicos **11**, **13**, e **15** (Fig. 2.9). Os resultados experimentais obtidos para a forma global "linear", onde apenas se encontrou o regioisómero 2'-hidroximetil-3'-aril-kielcorínico, contrastam com os descritos na literatura para a síntese dos análogos 4-metoxilados com esta forma global, a *trans*-(±)-kielcorina B (**34**) e a *trans*-(±)-isokielcorina B (**35**) [23]. A obtenção de ambos os regioisómeros foi explicada através da diferença de estabilidades dos dois radicais fenóxido possíveis para a 2,3-di-hidroxi-2-metoxixantona (**20**), precursor na síntese dos xantonolignóides **34-35** da série B [23].



(I) R1=R2=H, R3= O•,R4=OH
(II) R1=R2=H, R3=OH, R4= O•
(III) R1= O•, R2=OH, R3=R4=H
(IV) R1=OH, R2= O•, R3=R4=H
(V) R1=R4=H, R2= O•, R3=OH
(VI) R1=R4=H, R2=OH, R3=O•

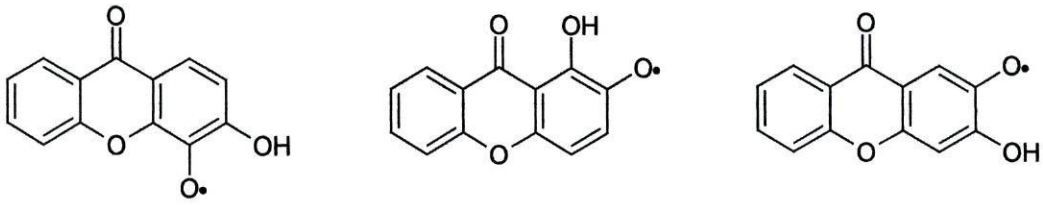
Fig. 2.9. Radicais possíveis para os blocos construtores xantónicos **11**, **13**, e **15**.

As energias associadas aos radicais formados foram calculadas com o intuito de correlacionar a estabilidade dos radicais intermediários **I-VI** (Fig. 2.9) com a natureza e a proporção dos derivados kielcorínicos formados. As energias totais absolutas ROHF/6-31G(d) são apresentadas na Tabela 2.1.

Tabela 2.1. Energias Calculadas (Valores Absolutos em Kcal.mol^{-1}) em Fase Gasosa Usando o GAMESS (*General Atomic and Molecular Electronic Structure System*) *ab-initio* [24]

Espécies	$E_{ROHF/6\text{-}31G(d)}$	$E_{ROHF/6\text{-}31G(d)}$ relativa à di-hidroxixantona (**a**)	Energia de dissociação estimada (**a-b**)	Diferença absoluta entre as energias de dissociação dos dois radicais possíveis
15	499752,7235	-	-	
11	499757,2401	-	-	
13	499753,3141	-	-	
I	499359,6568	393,0667	80,4206	2,6667
II	499362,3235	390,4000	77,7539	
III	499351,3012	405,9397	93,2936	18,1954
IV	499369,4966	387,7443	75,0982	
V	499364,0096	389,3045	76,6584	5,0571
VI	499358,9525	394,3616	81,7155	
	312,6461(**b**)	-	-	

Das energias de dissociação estimadas (**a-b**), é de esperar que a população relativa dos radicais resultantes obedeça aos seguintes padrões: **I** < **II**, **III** < **IV** e **V** > **VI**, com uma diferença de 2,7, 18,2 e 5,0 Kcal.mol^{-1}, respectivamente (Fig. 2.10).



II **IV** **V**

Fig. 2.10. Estimativa das populações mais abundantes das espécies radicalares.

É de notar que estes resultados representam parcialmente o sistema reaccional no qual os radicais estão envolvidos, não contabilizando factores estéricos, electrónicos ou solvente-dependentes, associados à abstração do átomo de hidrogénio. À semelhança da previsão anteriomente efectuada, estes dados teóricos conferem com os obtidos experimentalmente (***Anexos II*** e ***III***), isto é, na prática os regioisómeros mais abundantes foram os resultantes dos radicais fenóxido representados na Fig. 2.10. Especificando:

- na forma global "pistola", os compostos obtidos foram a *trans*- (**29**) e a *cis*-(±)-kielcorina C (**30**), resultantes do radical fenóxido **II**; a diferença significativa das energias dos dois radicais **II** e **I** da 3,4-di-hidroxixantona (**15**) (2,7 Kcal.mol^{-1}), pode ajudar a compreender o facto de na prática não se ter formado a isokielcorina C, que seria proveniente do radical menos estável (**I**);
- na forma global "long-chair" formou-se em maior quantidade (20-34%) o regioisómero *trans*-(±)-kielcorina D (**31**), resultante do radical fenóxido **IV**, e em muito menor quantidade (0,4-1,1%) o regioisómero *trans*-(±)-isokielcorina D (**32**), proveniente do radical fenóxido **III**, apesar da diferença das energias dos dois radicais **III** e **IV** da 1,2-di-hidroxixantona (**11**) ter sido a mais significativa (18,2 Kcal.mol^{-1}); este dado vem reforçar a hipótese desta reacção poder ser assistida pela formação de um complexo com o ião metálico;
- na forma global "linear", foi obtida a *trans*-(±)-kielcorina E (**33**), resultante do radical fenóxido **V**; a diferença significativa das energias dos dois radicais **V** e **VI** da 2,3-di-hidroxixantona (**13**) (5,0 Kcal.mol^{-1}), pode ajudar a compreender o facto de na prática não se ter formado a isokielcorina E, que seria proveniente do radical menos estável (**VI**).

Determinação Estrutural

A determinação estrutural da kielcorina **27**, inicialmente elucidada como uma 2,3,4-tri-hidroxixantona [25], ilustra a importância do desenvolvimento das técnicas de espectroscopia que, com a implementação das experiências bidimensionais de RMN, possibilitou de forma inequívoca a elucidação deste composto como a (±)-*trans*-3'-hidroximetil-2'-(4''-hidroxi-3''-metoxifenil)-1',4'-dioxano[5',6'-*c*]2-metoxixantona (***Anexo I***, pág. 99).

As estruturas dos derivados xantónicos sintetizados foram estabelecidas por IV, UV, técnicas de RMN, nomeadamente de ^{1}H e ^{13}C, correlação espectroscópica homonuclear (COSY) e heteronuclear (HETCOR, HMBC, HSQC), NOE e INEPT selectivo, EM de impacto electrónico e de alta resolução (***Anexos II***, ***V*** e ***VI***) e adicionalmente por cristalografia de raios X para os compostos **10**, **25** e **26** [26]. Os derivados xantónicos monoxigenados **2-7** e os dímeros do álcool coniferílico, álcool desidrodiconiferílico (**37**) e fenilcumarano (**38**), foram identificados por comparação com amostras autênticas por cromatografia em camada fina [23] e por análise espectral (ver experimental dos ***Anexos II, V,VI,VIII***).

A técnica de IV serviu para assegurar as transformações pretendidas na síntese dos compostos, assinalando os grupos funcionais característicos. Os espectros para compostos desta natureza apresentam bandas de absorção a 3500-3200 cm^{-1}, a 1660 cm^{-1} e a 1450 cm^{-1}, características de vibrações de estiramento das ligações O-H, C=O e C=C do anel aromático, respectivamente [27].

Igualmente, a espectrofotometria de UV, por ter sido anteriormente usada de uma forma extensiva na elucidação estrutural de derivados xantónicos [22,28], forneceu uma resposta rápida sobre a localização dos grupos hidroxilos das *orto*-di-hidroxixantonas. Esta classe de compostos revela um espectro de UV característico, normalmente com quatro bandas entre 200 e 400nm de intensidade decrescente [29]. Os valores da análise espectrofotométrica de UV para os compostos **11**, **13** e **15** não foram apresentados nos anexos correspondentes por estarem de acordo com os dados descritos na literatura. Exemplificando na Fig. 2.11, o espectro em etanol para a 2,3-di-hidroxixantona (**13**), sugeriu que este composto corresponderia a uma xantona [22,28]. A adição de hidróxido de sódio ionizou os grupos hidroxilo em todas as posições do esqueleto xantónico, resultando em profundas alterações no espectro de absorção; o composto **13** originou uma banda intensa a 345-365 nm, característica de uma xantona com um hidroxilo em C(3), em posição *para* ao carbonilo, em consequência do seu maior carácter ácido [22]; a adição de ácido clorídrico à solução alcalina permitiu

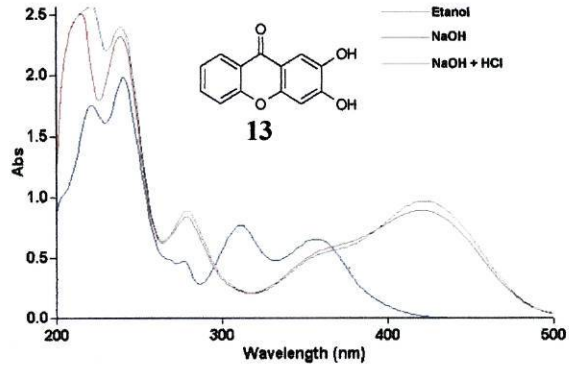


Fig. 2.11.

Espectros da 2,3-di-hidroxixantona (**13**) em etanol (——), em etanol e hidróxido de sódio (NaOH) (——) e em etanol, NaOH e ácido clorídrico (HCl) (——).

verificar que o composto **13** não é estável (Fig. 2.11, o espectro não retomou o traçado inicial) sob condições básicas, característica de xantonas com sistemas *orto* ou *para*-quinólicos, que se decompõem na presença de hidróxido de sódio. Verificou-se um comportamento homólogo para a 1,2- (**11**) e 3,4-di-hidroxixantona (**15**). O cloreto de alumínio por formar complexos ácido-lábeis com grupos *orto*-di-hidroxilo, pode também ser usado para caracterizar estes compostos [22]. Os efeitos bactocrómicos no espectro de UV, causados pela adição de cloreto de alumínio, exemplificados na Fig. 2.12 para a 1,2-di-hidroxixantona (**11**), são característicos de xantonas com grupos *orto*-di-hidroxilo ou com um grupo hidroxilo adjacente ao carbonilo da xantona, nas quais se observa uma redução forte da intensidade e um efeito bactocrómico de cerca de 20nm do segundo principal máximo de absorção [22]; o espectro tomaria o aspecto inicial com a adição de ácido clorídrico, evidenciando a presença de um grupo *orto*-di-hidroxilo, observação que foi verificada para a 2,3- (**13**) e 3,4-di-hidroxixantona (**15**). No entanto, no caso da 1,2-di-hidroxixantona (**11**), devido à existência de um grupo hidroxilo adjacente ao carbonilo, formou-se um complexo ácido-estável com o cloreto de alumínio (Fig. 2.12, o espectro não retomou o estado inicial).

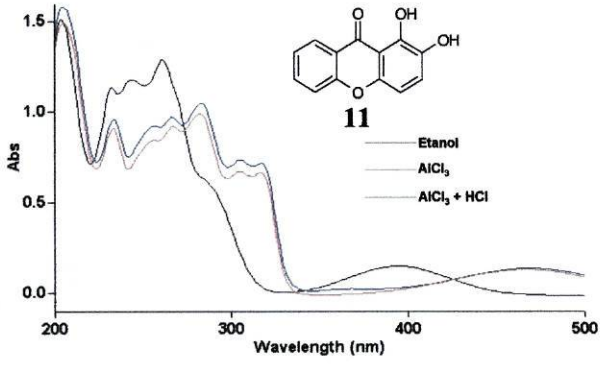


Fig. 2.12.

Espectros da 1,2-di-hidroxixantona (**11**) em etanol (——), em etanol e cloreto de alumínio ($AlCl_3$) (——) e em etanol, $AlCl_3$ e HCl (——).

Os espectros de RMN de ^{1}H e ^{13}C permitiram não só confirmar as transformações pretendidas, como elucidar de forma inequívoca os compostos sintetizados. Os assinalamentos de todos os protões dos compostos sintetizados foram conseguidos com base na multiplicidade dos sinais e nas constantes de acoplamento. Os espectros de RMN de ^{13}C obtidos para os derivados xantónicos apresentam valores de desvio químico que estão de acordo com a estrutura proposta para estes compostos [30]. A título exemplificativo, na Fig. 2.13 é possível observar os sinais correspondentes aos protões e carbonos do anel aromático não substituído do núcleo xantónico, que aparecem com desvios químicos característicos.

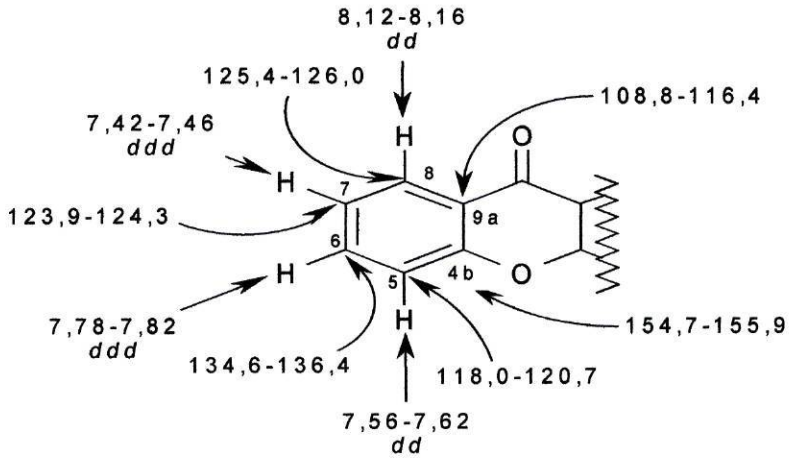
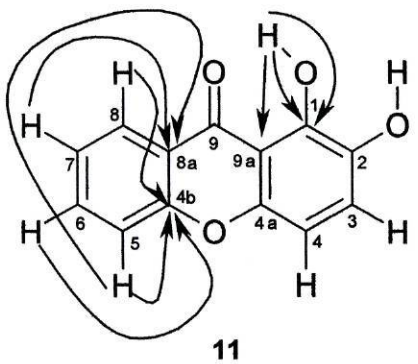


Fig. 2.13. Intervalos dos desvios químicos para os derivados xantónicos sintetizados.

Técnicas de INEPT selectivo e HETCOR permitiram determinar e/ou corrigir elucidações estruturais de xantonas simples oxigenadas anteriormente descritas (ver experimental de ***Anexos II*** e ***V***). Exemplificando para a 1,2-di-hidroxixantona (**11**), os assinalamentos dos sinais correspondentes aos carbonos C(1) C(4b), C(8a) e C(9a) foram efectuados através de INEPT selectivo (Fig. 2.14), visto que: irradiando o H-C(1) foi observado um aumento dos sinais correspondentes aos carbonos C(1) e C(9a); irradiando o H-C(5) foi observado um aumento dos sinais correspondentes aos carbonos C(4b) e C(8a);



11

Fig. 2.14.
Principais conectividades encontradas para a 1,2-di-hidroxixantona (**11**) nas experiências de INEPT selectivo.

irradiando o H-C(6) foi observado um aumento do sinal correspondente ao carbono C(4b); irradiando o H-C(7) foi observado um aumento do sinal correspondente ao carbono C(8a); irradiando o H-C(8) foi observado um aumento do sinal correspondente ao carbono C(4b).

O ***Anexo II*** (pág. 108-123) descreve a determinação estrutural dos xantonolignóides **29-33** e demonstra como as experiências de NOE e de INEPT selectivo são técnicas imprescindíveis na determinação inequívoca da estereoquímica e da orientação dos substituintes do anel 1,4-dioxano dos xantonolignóides, respectivamente.

Os espectros de massa dos derivados xantónicos possuem padrões de fragmentação característicos, caracterizando-se pela presença de picos moleculares relativamente intensos, por processos gerais de expulsão consecutiva de etino (clivagem-β) e por fragmentação-α; esta última, devido à presença de carbonilo entre dois anéis aromáticos, ocorre em proporção relativa considerável [31]. O exemplo na Fig. 2.15 para a *trans*-(±)-isokielcorina D (**32**) põe estes factos em evidência. Após análise dos espectros de EM para os isómeros xantónicos e

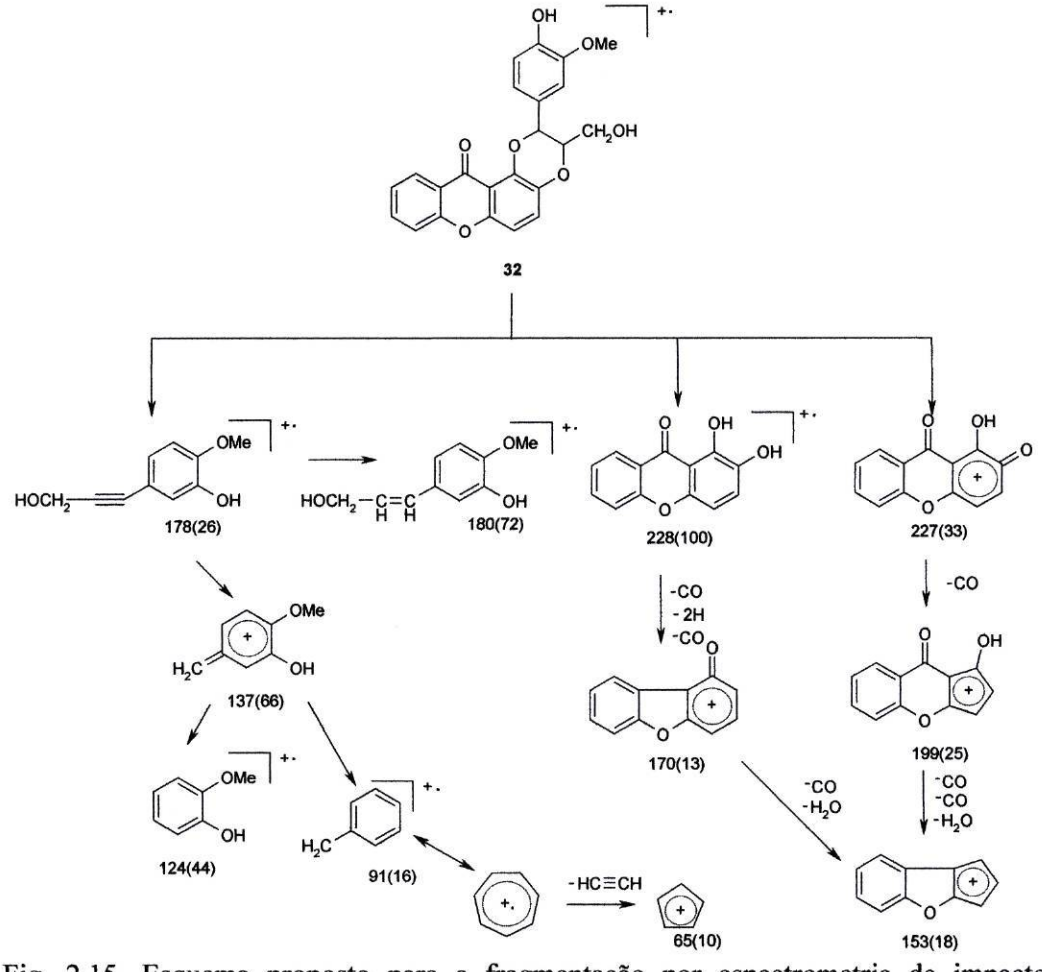


Fig. 2.15. Esquema proposto para a fragmentação por espectrometria de impacto electrónico para a *trans*-(±)-isokielcorina D (**32**).

kielcorínicos foi verificado que a fragmentação difere essencialmente na relação de intensidade dos picos e no pico base.

A cristalografia de raios X é outro método poderoso de determinação estrutural com a capacidade única de elucidar, de forma inequívoca, a estrutura molecular tridimensional de substâncias cristalinas. Foram já determinadas por este processo, as estruturas de vários derivados xantónicos [32-65], nomeadamente da 1,2-dimetoxixantona (**10**) e dos seus precursores de síntese, o 2-(3,4-dimetoxifenoxi)-benzoato de metilo (**25**) e o ácido 2-(3,4-dimetoxifenoxi)-benzóico (**26**) [26].

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Capítulo 3

Resolução Enantiomérica de Xantonolignóides

Most natural organic products, the essential products of life, are assymetric and possess such asymmetry that they are not superimposable on their images.

Louis Pasteur
aquando da primeira separação de cristais de enantiómeros de um sal de ácido tartárico, 1848

Provavelmente o melhor exemplo da influência da quiralidade é dado pela Natureza. A maioria dos sistemas de reconhecimento molecular na natureza (enzimas, receptores) distingue enantiómeros.

Um exemplo paradigmático da influência da quiralidade de fármacos é o da tragédia da talidomida, um fármaco administrado na forma racémica às mulheres grávidas na década de sessenta para aliviar os enjôos nos primeiros meses de gravidez, e que foi responsável por malformações severas nos recém-nascidos. Apesar dos estudos iniciais indicarem que a acção teratogénica adviria do enantiómero (*S*)-(-), possuindo o enantiómero (*R*)-(+) a acção indutora do sono, estudos mais recentes, demonstraram que os enantiómeros da talidomida sofrem rápida racemização *in vivo*, tornando difícil afirmar que a toxicidade se deve apenas a um dos enantiómeros [1]. Assim, diferentes enantiómeros podem produzir diferentes comportamentos, quer farmacocinéticos (ex. diferentes velocidades de metabolização), quer farmacodinâmicos (ex. diferentes afinidades para o receptor). Devido às implicações destas diferenças, os métodos analíticos capazes de resolver racematos são cruciais para os ensaios clínicos e toxicológicos de fármacos [2].

Separação de Enantiómeros

Os métodos analíticos usados na separação enantiomérica directa incluem a cromatografia líquida de alta resolução (HPLC), a cromatografia em camada fina, a cromatografia gasosa, a cromatografia de fluído supercrítico e a electroforese capilar [3]. Enquanto que a cromatografia gasosa está mais ou menos restrita a compostos mais voláteis, o método de eleição para a separação de compostos mais polares, como o são a maioria dos fármacos, é a metodologia por HPLC, na qual se destaca a diversidade de fases quirais desenvolvidas [4]. Vários autores classificaram as fases estacionárias quirais de acordo com o mecanismo de reconhecimento quiral estabelecido. Segundo Taylor e Maher [5], as fases estacionárias quirais podem ser divididas do seguinte modo:

Tipo 1: O soluto faz parte de um complexo metálico diastereoisomérico (cromatografia de troca de ligante).

Tipo 2: As únicas interacções essencialmente discerníveis são as interacções atractivas de transferência de carga.

Tipo 3: O complexo soluto-fase estacionária quiral é formado por múltiplas interacções atractivas, incluindo pontes de hidrogénio, interacções dipolo-dipolo e empilhamento π–π, entre o soluto e uma fase estacionária quiral de baixo peso molecular. São normalmente designadas de fases estacionárias quirais de Pirkle.

Tipo 4: Ocorrem interacções entre o soluto e a fase estacionária, seguidas da inclusão em cavidades quirais numa fase estacionária de peso molecular relativamente baixo (ex° ciclodextrinas).

Tipo 5: O mecanismo primário para a formação do complexo soluto-fase estacionária quiral é através de interacções atractivas mas a inclusão possui também um papel importante devido a estas fases estacionárias quirais serem polímeros de elevada massa molecular (ex° polissacarídeos de celulose e amilose).

Tipo 6: A fase estacionária quiral é uma proteína e os complexos soluto-fase estacionária quiral são baseados na combinação de interacções hidrofóbicas e polares. Este grupo pode ser considerado como uma subdivisão do Tipo 5.

Nas fases estacionárias quirais do tipo 5, os derivados de carbamatos têm sido muito usados pela sua elevada enantiosselectividade face a uma grande variedade de classes de compostos [6-9].

O ***Anexo IV*** (pág. 138-158) descreve a separação analítica por HPLC quiral dos enantiómeros dos derivados kielcorínicos **29-33**, no qual é apresentado um estudo comparativo em colunas quirais de carbamatos derivados de polissacarídeos de amilose e celulose, é investigada a influência das fases móveis na resolução destes compostos e é ilustrado o potencial do método de eluição múltiplo. Neste trabalho, os resultados obtidos foram relacionados com a forma global dos xantonolignóides, que pode originar diferenças no *fit* dos pares de enantiómeros na cavidade quiral das fases estacionárias quirais.

Os mecanismos para o reconhecimento quiral e para as interacções entre os enantiómeros e as fases estacionárias quirais do tipo 5, fenilcarbamatos derivados de polissacarídeos, são descritos em termos de ligações de hidrogénio, interações dipolo-dipolo e interações π–π [9]. Nos xantonolignóides **29-33**, os grupos CH_2OH e C=O podem estabelecer ligações de hidrogénio com os grupos C=O e N-H, respectivamente, das fases estacionárias quirais, podendo também ser estabelecidas ligações electrostáticas do tipo dipolo-dipolo. Adicionalmente, interacções π–π entre os grupos aromáticos das fases estacionárias quirais e dos xantonolignóides **29-33** podem ter um papel importante no reconhecimento quiral.

Na Fig. 4.1 é representada uma proposta das interacções estabelecidas entre os xantonolignóides e as fases estacionárias quirais de fenilcarbamatos derivados de polissacarídeos.

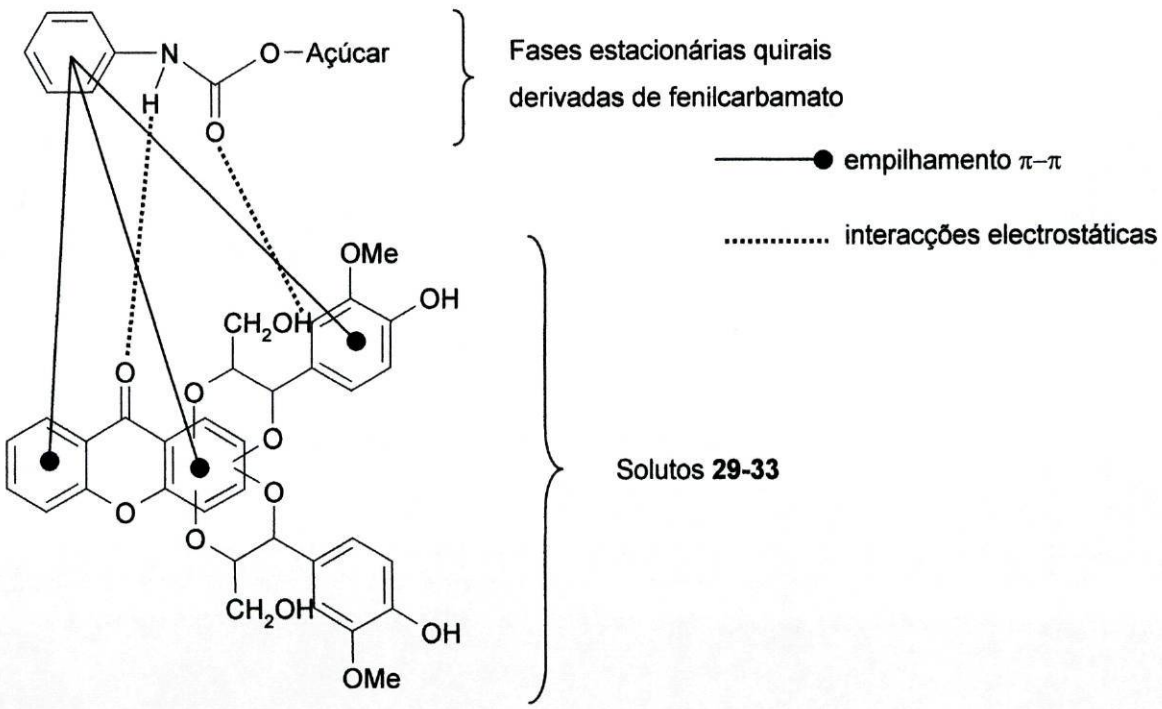


Fig. 4.1. Interacções mais prováveis entre os xantonolignóides e as fases estacionárias quirais utilizadas.

Cromatografia Semipreparativa por HPLC de Xantonolignóides em Fase Estacionária Quiral de Polissacarídeo de Amilose com Eluição Polar-Orgânica

INTRODUÇÃO

A obtenção de substâncias homoquirais é fundamental para a potência, selectividade, estabilidade e segurança dos fármacos. Adicionalmente, o estudo da actividade de isómeros ópticos puros pode fornecer dados sobre a natureza do receptor biológico [10]. Pelo exposto, decidiu efectuar-se a resolução das misturas racémicas *trans*-(±)-kielcorina C (**29**), da *trans*-(±)-kielcorina D (**31**), da *trans*-(±)-isokielcorina D (**32**) e da *trans*-(±)-kielcorina E (**33**) em escala semi-preparativa, para posterior avaliação das actividades biológicas dos seus enantiómeros.

Os resultados analíticos (***Anexo IV***) demonstraram que a fase estacionária quiral *tris*-(3,5-dimetilfenilcarbamato) de amilose seria a mais indicada para uma escala preparativa, na medida em que permite uma separação completa de cada um dos enantiómeros dos compostos **31-33** e uma separação parcial do composto **29**. De igual modo, os baixos tempos de retenção obtidos para estes compostos com o uso de solventes orgânicos polares (***Anexo IV***) levou à escolha deste tipo de eluição, em detrimento de fases móveis mais tradicionais.

MATERIAL E MÉTODOS

Os solventes, metanol, acetonitrilo, etanol e propan-2-ol foram produtos "*gradient grade* para cromatografia em fase líquida" da firma Merck (Darmstadt, Alemanha). As fases móveis foram previamente desgasificadas por um período de 15 minutos num banho de ultra-sons. Os eluentes usados foram sempre medidos na relação volume/volume.

A cromatografia analítica foi realizada num cromatógrafo System SMI Pump Series II (Gloucester, UK) equipado com um injector Rheodyne 7125 com um reservatório de 20 μL, utilizando um detector de diodos TSP-UV6000LP e um integrador Chromquest para Windows NT. A análise foi realizada a 25°C, em trabalho isocrático, a um fluxo de 0,4 $mL.min^{-1}$, numa coluna de *tris*-3,5-dimetilfenilcarbamato de amilose (20% (m/m)) adsorvida em aminopropilsílica (APS)-Nucleosil (500 Å, 7 μm) (15 x 0,46 cm diâmetro interno). A avaliação da pureza enantiomérica foi analisada em triplicatas.

A cromatografia preparativa decorreu num cromatógrafo modelo 880 PU (Jasco, Japão) equipado com um injector Rheodyne 7125 com um reservatório de 334 μL, um detector 875/UV (Jasco, Japão) e um integrador CSW 1.7. A análise foi realizada a 25°C, em trabalho isocrático, a um fluxo de 1,8 $mL.min^{-1}$, numa coluna de *tris*-3,5-dimetilfenilcarbamato de amilose (20% (m/m)) adsorvida em APS-Nucleosil (500 Å, 7 μm) (20 x 0,7 cm diâmetro interno). Os volumes mortos das colunas foram considerados iguais à frente do solvente e determinados para cada análise em particular. As colunas foram preparadas pelo Laboratório de Síntese Orgânica e CLAE, Departamento de Química-UFSCar de acordo com o procedimento descrito [*] [11,12]. Uma amostra de cada composto, foi pesada e dissolvida em metanol. As soluções resultantes foram injectadas no sistema cromatográfico e foram colectadas as fracções do primeiro e do segundo enantiómero. A fase móvel das fracções obtidas foi evaporada com o auxílio de pressão reduzida e os sólidos obtidos foram

[*] Colunas preparadas pela Professora Doutora Elizabeth Tiritan e pela Drª Regina Oliveira sob orientação da Professora Doutora Quézia Cass.

analisados por HPLC para determinação da pureza enantiomérica. A rotação óptica dos compostos foi determinada num polarímetro Polartronic Universal (Schmidt + Haensch).

O factor de retenção (k_1) do primeiro enantiómero a eluir foi determinado como [$k_1 = (t_{R1}-t_0)/t_0$] no qual t_R corresponde ao tempo de retenção do primeiro enantiómero e t_0 ao tempo-morto da coluna. O factor de separação (α) foi calculado como [$\alpha = k_2-k_1$] e a de resolução (R_s) como [$R_s = 2\ (t_{R2}-t_{R1})/(W_1+W_2)$] sendo W_i a largura do pico na base de cada enantiómero.

RESULTADOS E DISCUSSÃO

As análises foram efectuadas com soluções saturadas dos compostos **29**, **31-33**. Na Tabela 4.1 encontram-se descritos os valores dos desvios da rotação óptica para os enantiómeros.

Tabela 4.1. Desvios da Rotação Óptica Para os Enantiómeros dos Compostos **29**, **31-33**

Racemato	**Ordem de Eluição**	$[\alpha]^{20}_D$ (metanol) $c = 0{,}33$
29	Primeiro	-3
	Segundo	+3
31	Primeiro	+5
	Segundo	-4
32	Primeiro	-5
	Segundo	+6
33	Primeiro	+3
	Segundo	-3

*Composto **29***

Uma quantidade de 6,6 mg foi submetida à separação semi-preparativa. O perfil da separação analítica é mostrada na Fig. 4.2. Este método produziu um tempo de retenção curto e uma separação parcial ($k_1 = 0{,}75$; $\alpha = 1{,}3$; $Rs = 1{,}0$). O método analítico foi modificado para uma escala semi-preparativa (Fig. 4.3). A separação de base foi conseguida em 12 min. Uma

pureza de 99% de excesso enantiómerico foi conseguida para o primeiro enantiómero (-) e de 96% para o segundo enantiómero (+) com um rendimento de 48% para o primeiro enantiómero (-) e de 36% para o segundo (+).

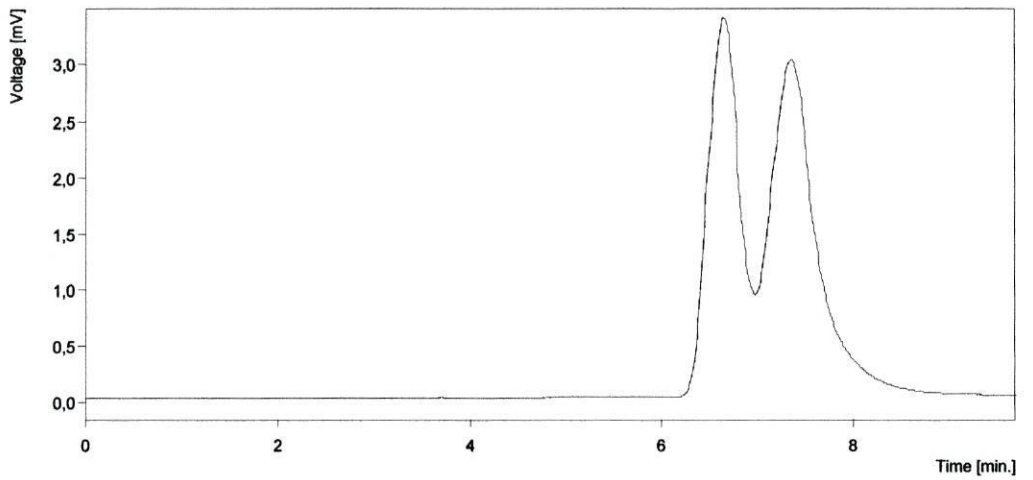


Fig. 4.2. Separação analítica do composto **29**. Análise conduzida na FEQ a 239 nm. Fase móvel: propan-2-ol-etanol (3:7), fluxo 0,4 mL.min^{-1}.

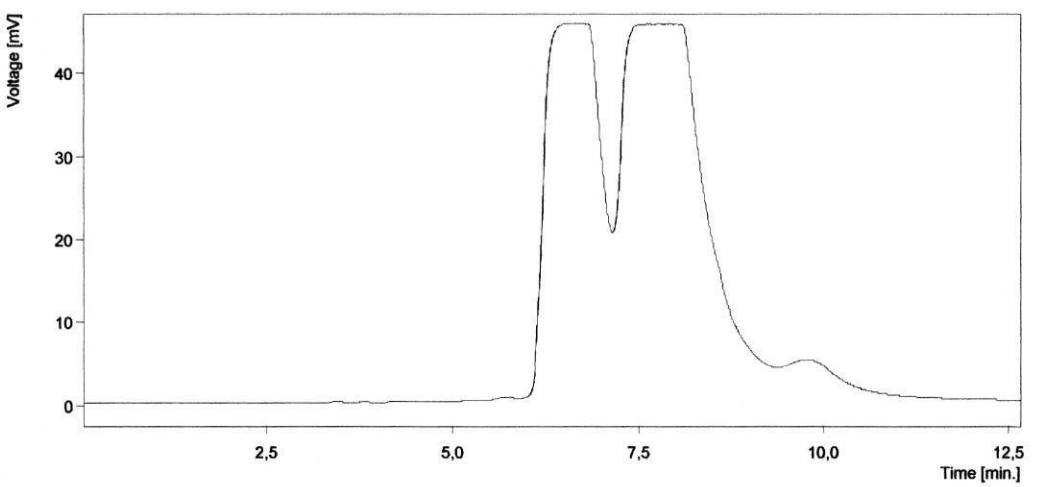


Fig. 4.3. Separação semi-preparativa do composto **29**. Análise conduzida na FEQ a 340 nm. Fase móvel: propan-2-ol-etanol (3:7), fluxo 1,8 mL.min^{-1}.

*Composto **31***

Uma quantidade de 3,5 mg foi submetida à separação semi-preparativa. O perfil da separação analítica é mostrada na Fig. 4.4. Este método produziu um tempo de retenção curto e uma boa separação (k_1 = 1,9; α = 1,6; *Rs* = 2,0). O método analítico foi modificado para uma escala semi-preparativa (Fig. 4.5). A separação de base foi conseguida em 16 min. Uma pureza de >99% de excesso enantiómerico foi conseguida para o primeiro enantiómero (+) e de 99% para o segundo enantiómero (-) com um rendimento de 86% para ambos.

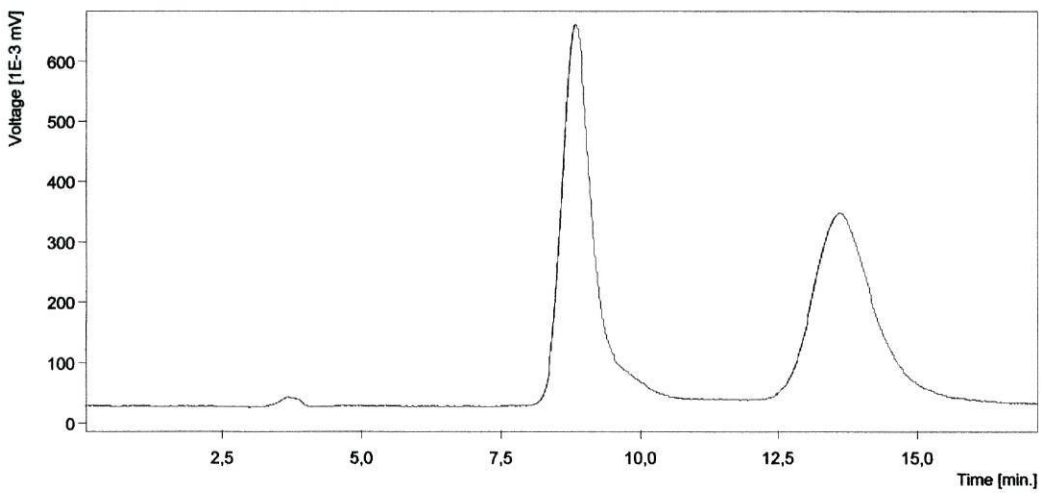


Fig. 4.4. Separação analítica do composto **31**. Análise conduzida a 239 nm. Fase móvel: metanol-etanol (5:5), fluxo 0,4 mL.min^{-1}.

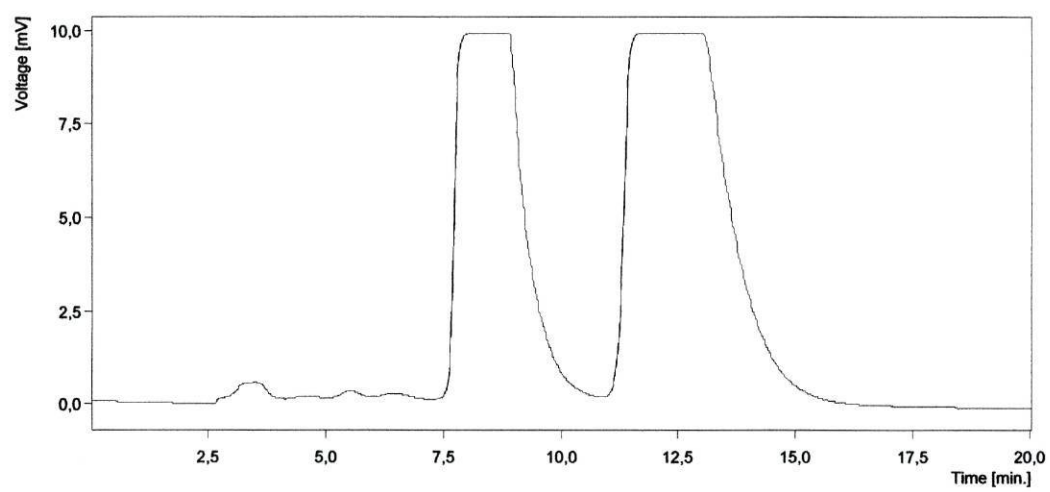


Fig. 4.5. Separação semi-preparativa do composto **31**. Análise conduzida a 239 nm. Fase móvel: metanol-etanol (5:5), fluxo 1,8 mL.min^{-1}.

Composto ***32***

Uma quantidade de 5,1 mg foi submetida à separação semi-preparativa. O perfil da separação analítica é mostrada na Fig. 4.6. Este método produziu um tempo de retenção curto e uma boa separação (k_1 = 4,0; α= 1,9; *Rs* = 3,0). O método analítico foi modificado para uma escala semi-preparativa (Fig. 4.7). A separação de base foi conseguida em 14 min. Uma pureza de >99% de excesso enantiómerico foi conseguida para os dois enantiómeros com um rendimento de 58% para o primeiro enantiómero (-) e de 80% para o segundo (+).

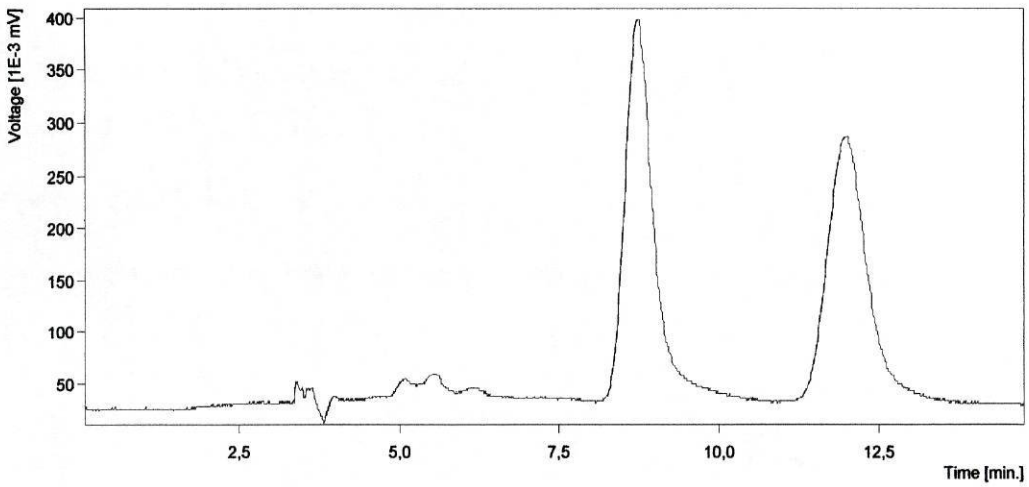


Fig. 4.6. Separação analítica do composto **32**. Análise conduzida a 239 nm. Fase móvel: metanol-etanol (5:5), fluxo 0,4 mL.min^{-1}.

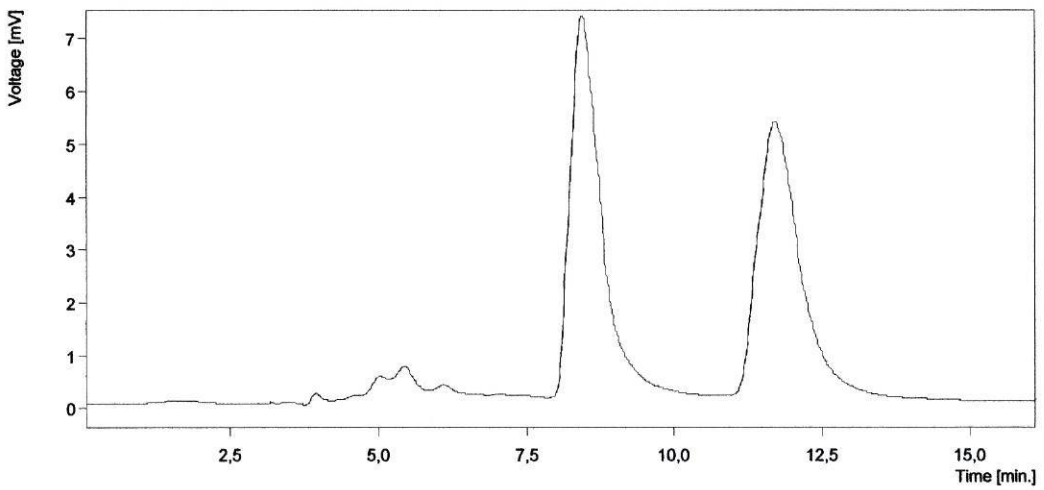


Fig. 4.7. Separação semi-preparativa do composto **32**. Análise conduzida a 340 nm. Fase móvel: metanol-etanol (5:5), fluxo 1,8 mL.min^{-1}.

Composto 33

Uma quantidade de 4,4 mg foi submetida à separação semi-preparativa. O perfil da separação analítica é mostrada na Fig. 4.8. Este método produziu um tempo de retenção curto e uma boa separação (k_1 = 1,1; α = 2,2; *Rs* = 4,0). O método analítico foi modificado para uma escala semi-preparativa (Fig. 4.9). A separação de base foi conseguida em 17 min. Uma pureza de >99% de excesso enantiómerico foi conseguida para os dois enantiómeros com um rendimento de 90% para o primeiro enantiómero (+) e de 86% para o segundo (-).

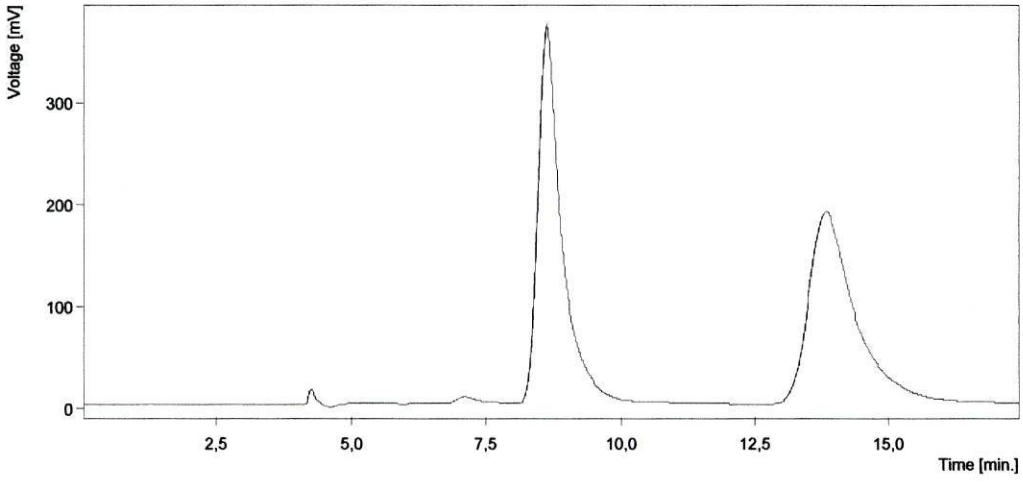


Fig. 4.8. Separação analítica do composto **33**. Análise conduzida a 242 nm. Fase móvel: metanol-acetonitrilo (5:5), fluxo 0,4 mL.min^{-1}.

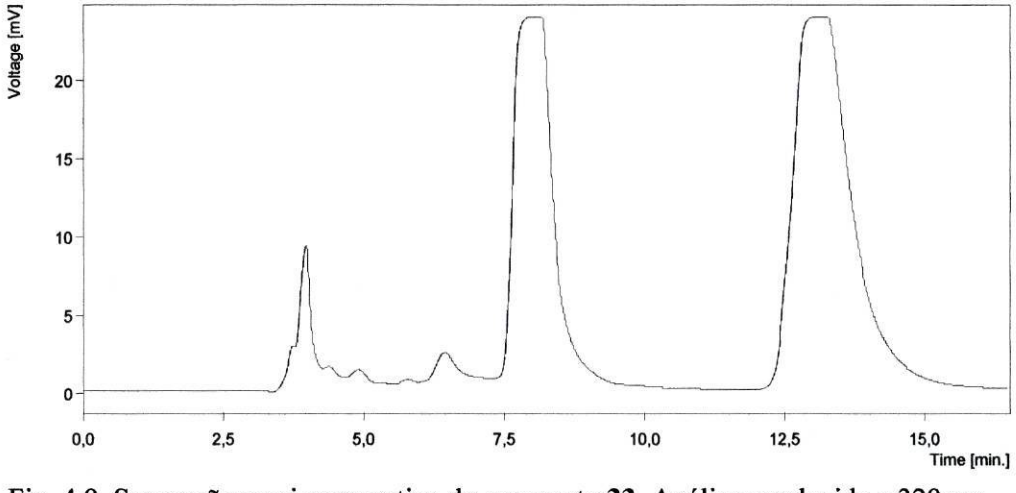


Fig. 4.9. Separação semi-preparativa do composto **33**. Análise conduzida a 329 nm. Fase móvel: metanol-acetonitrilo (5:5), fluxo 1,8 mL.min^{-1}.

A utilização de solventes mais polares diminuiu os problemas de solubilidade para estes compostos, encontrados quer em fases móveis de álcoois-hidrocarbonetos, quer em fases móveis de álcoois-água. Outras vantagens da eluição em modo polar-orgânico foram: a diminuição dos tempos de retenção com o consequente aumento da produtividade a uma escala semi-preparativa e o uso de solventes com pontos de ebulição mais baixos tornando mais fácil o processo de recuperação do enantiómero.

A avaliação na inibição do crescimento celular pelos enantiómeros obtidos será abordada no Capítulo 4.

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Capítulo 4

Actividades Biológicas e Estudos de Relação Estrutura-Actividade

Man can't help hoping even if he is a scientist. He can only hope more accurately.

Karl Memmiger [1]

Novos agentes quimioterápicos com novos mecanismos bioquímicos para o tratamento do cancro em geral e da resistência tumoral em particular são uma necessidade permanente no arsenal terapêutico [2]. Nesta área, é urgente o desenvolvimento de uma nova classe de compostos com actividade antitumoral, que permita reduzir os efeitos secundários dos fármacos antineoplásicos disponíveis [3,4]. Durante as últimas décadas os estudos em compostos naturais têm sido particularmente bem sucedidos neste campo de investigação [5,6]. Nomeadamente espécies da família Guttiferae (Clusiaceae) têm revelado compostos com um interesse considerável em bioensaios de *screening* [7]. Dos fármacos aprovados entre 1983 e 1994 pela *Food and Drug Administration* (FDA) como antineoplásicos, 61% correspondiam a produtos naturais ou a produtos de síntese baseados em modelos de produtos naturais [8].

Neste enquadramento foi avaliada a actividade antitumoral e imunomoduladora dos xantonolignóides (***Anexo II***) e das xantonas simples oxigenadas (***Anexo V***) e também a modulação da PKC (***Anexos VI-VIII***). A selecção das três actividades biológicas foi baseada na experiência do grupo do CEQOFFUP, nos dados encontrados na literatura desta classe de compostos e, fundamentalmente, na possibilidade de interligação das três respostas biológicas. Sendo a PKC uma família de enzimas envolvida quer na promoção tumoral e mitogénese, quer em processos de secreção e inflamação, poderá encontrar-se intimamente ligada à actividade antitumoral e imunomoduladora [9-13]. Por sua vez, um exemplo de um derivado xantónico que ilustra a estreita relação entre estas últimas actividades, é a do ácido 5,6-dimetilxantenon-4-acético (DMXAA, **17**), que causa necrose de tumores por imunomodulação [14].

Actividade Sobre o Crescimento de Linhas Celulares Tumorais Humanas

Das muitas actividades descritas para as xantonas, sobressai o seu efeito inibidor do crescimento *in vitro*, de uma larga variedade de linhas celulares tumorais. Estas incluem células leucémicas [15-25], células de carcinoma epidermóide oral [26-32], melanoma [21,33], células do carcinoma do cólon [18,19,21,27,34-40], da mama [18,19,36], do ovário [21,39], da próstata [21], do pulmão [16,18,21,25,41,42], do fígado[42], do estômago [42], do carcinoma renal [21], neuroblastomas [43], células adrenocorticais [44,45], fibroblastos [35,46], células epiteliais [46,47], células tumorais de Friend [23], células de hepatoma [28,29,48] e células do carcinoma da bexiga [48]. Igualmente foram

realizados estudos *in vivo* em adenocarcinoma do cólon [49-55], leucemia linfocítica [26,32,56,57]e tumores xenográficos de ovário, melanoma [54], formas de tumores sólidos sarcoma 180 [56,58,59], carcinoma de Ehrlich [40,58] e glioma [40].

As xantonas descritas como tendo actividade antitumoral incluem para além da xantona (**1**) [38], outros produtos naturais e de síntese. Estruturalmente, os compostos naturais investigados dividem-se em: xantonas simples oxigenadas [28,36,43] e glicosiladas [59], xantonolignóides [18], xantonas preniladas [17,27,30-32,34,39,46] e relacionadas como furanoxantonas[18,36] e benzopiranoxantonas [27,30], bixantonas [16], xantonas mistas como xantonas-antraquinonas [44,45] e uma xantona-cromona [35] e xantonas policíclicas [47]. Para os compostos obtidos por síntese são indicadas as modificações moleculares na Tabela 3.1, tendo como modelos estruturas de compostos com actividade citotóxica previamente estabelecida.

Tabela 3.1. Substituintes Localizados no Núcleo Xantónico ou em Isósteros Associados à Actividade Citotóxica

Substituintes	**Modelos**
Ácido acético [40,41,49-53]	ácido flavonoacético
Anéis benzopirano [22,24,58]	psorospermina (**18**) e benzo[*b*]acronicina
Anéis furano [57]	5-metoxiesterigmatocistina
Azatioxantonas [23,56,60] com substituintes piridínilo [56,60]	lucantona, azaelipticina e azaantraquinona
Cadeia carboxamida [56]	aminoacridina-2-carboxamida
Cadeia 4-oxo-2-furanilbuteniloxi [20,33]	geiparvarina
Epóxidos [28,29,41,48]	prenilflavonas e psorospermina (**18**) (furanoxantona)
α-Metiliden-γ-butirolactona [21]	variedade de fármacos antineoplásicos
Piperazinil e flúor [37]	fluoroquinolonas

A influência de alguns derivados xantónicos em processos celulares relacionados com a tumorogénese e com a interação em alvos celulares importantes para a actividade antitumoral foi sumarizada na Tabela 3.2.

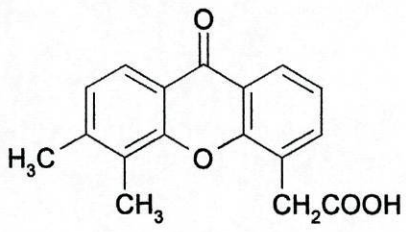
Tabela 3.2. Mecanismos de Acção Avaliados Para os Derivados Xantónicos com Actividade Citotóxica

Mecanismos	**Derivados Xantónicos**
Bloqueio dos receptores da prostaglandina E2	xantona carboxilada [41] e xantona prenilada [61]
Indução da apoptose:	
pela activação da caspase-3	xantonas preniladas [15]
pela inibição da esfingomielinase	xantonas preniladas [62,63]
Imunomodulação e indução de citoquinas	DMXAA (**17**) [14,54,55,64-70]
Inibição da aromatase e da 17-α-hidroxilase/C17,20-lipase	imidazolxantonas e triazolxantonas [71]
Inibição da fosfolipase C	xantona-cromona [35] e xantona simples polioxigenada [72]
Inibição da 11-β-hidroxilase	xantona-antraquinona [44]
Inibição das topoisomerases I e II	xantonas simples oxigenadas, preniladas, xantonolignóides [73], benzopiranoxantonas [73] e furanoxantonas [73]
Inibição da transdução do sinal na cascata dos factores de crescimento pela repressão específica do oncogene Ha-ras	xantonas com epóxidos [29,48]
Interferência na biossíntese:	
do DNA	xantonas policíclicas [29,47,48] e xantona prenilada[a, 42]
do RNA	xantonas com epóxidos [29,48]
protéica	xantonas com epóxidos [29,48]
Interferência na reparação pós-replicação	xantonas simples polioxigenadas [74]
Modulação das quinases:	
A de proteínas (PKA)	xantonas preniladas [75]
C de proteínas (PKC)	xantonas preniladas [75,76] e simples polioxigenadas [77,78]
IkappaB (IKK)	ácido 5,6-dimetilxantenon-4-acético (DMXAA, **17**) [64]
Quebras no DNA e *cross-links* DNA-proteínas	furanoxantona psorospermina (**18**)[79]

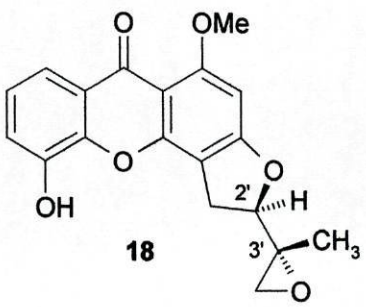
[a] correlação entre mecanismo/composto não foi observada.

Adicionalmente ao efeito antiproliferativo, alguns derivados xantónicos foram também descritos pelas suas propriedades antimutagénicas [74] e quimiopreventivas de cancro, como inibidores de promotores tumorais quer *in vitro* [80,81,82], quer *in vivo* [83].

Destacam-se pela rápida evolução no campo dos fármacos antineoplásicos, dois derivados xantónicos, o DMXAA (**17**) e a psorospermina (**18**).



17



18

O primeiro foi obtido por síntese [53] e os ensaios clínicos de fase I decorreram no Reino Unido e na Nova Zelândia [84]. O DMXAA (**17**) causa um rápido colapso vascular e a necrose dos tumores por imunomodulação e indução de citoquinas, particularmente do factor de necrose tumoral [14,54,55,64-66], do interferão-gama [67] e da proteína 10 interferão-induzida [55]; possui efeitos indutivos na 5-hidroxitriptamina [68], no óxido nitroso [69,70] e na indução de factores de transcrição [64,67], tendo demonstrado efeitos sinérgicos, como adjuvante na radioterapia [85]. Na avaliação do perfil farmacocinético do DMXAA (**17**), foram realizados estudos de distribuição-metabolismo [86-89] e foi já avaliada a interacção com outros fármacos[90,91].

O composto **18** é uma di-hidrofuranoxantona natural, isolada das raízes e cascas da planta tropical africana *Psorospermum febrifugum* [26,92], que mostrou resultados promissores como agente antileucémico [26]. A psorospermina (**18**) é activa contra linhas celulares tumorais humanas da mama, do colon, da leucemia linfocítica [19], de leucemias fármaco-resistentes [93] e no linfoma SIDA-relacionado [93]. A estrutura e a estereoquímica do composto **18**, como a de vários análogos, é já conhecida, tendo sido determinada a importância da configuração e da funcionalidade do grupo epoxidi-hidrofurano para a actividade *in vivo* [36,94,95]. Estudos mecanísticos demonstraram que a psorospermina (**18**) actua por intercalação na molécula do DNA posicionando o seu cromóforo tricíclico, o núcleo xantónico, numa orientação paralela com os pares de bases do DNA, e por modificação covalente do N(7) da guanina através do epóxido [79,96,97] e mediada pela topoisomerase II [98,99]. O intermediário da reacção topoisomerase II-DNA é aprisionado e a enzima topoisomerase II é impedida de catalizar

a reacção de quebra-reunião das cadeias do DNA [99,100].

Considerando a actividade citotóxica descrita para xantonas naturais com uma estrutura simples polioxigenada e os resultados promissores do fármaco antineoplásico DMXAA (**17**), decidiu-se proceder ao estudo da actividade das xantonas sintetizadas no crescimento de várias linhas celulares tumorais humanas, com o objectivo de estabelecer uma relação estrutura-actividade, já que não existem estudos deste tipo com xantonas simples oxigenadas. No ***Anexo V*** (pág. 159-165) descrevem-se os resultados e algumas considerações sobre a relação da estrutura de vinte e sete xantonas oxigenadas, todas sintetizadas no CEQOFFUP e a actividade em três linhas celulares tumorais humanas: MCF-7 (adenocarcinoma da mama), TK-10 (carcinoma renal) e UACC-62 (melanoma) [*].

A presença de grupos hidroxilo do tipo catecol em algumas xantonas simples oxigenadas, pode explicar a relação estrutura-actividade apresentada no ***Anexo V***. A hipótese de xantonas *orto*-di-hidroxiladas se poderem transformar em quinonas reactivas pode justificar o aumento na sua actividade antitumoral em relação a xantonas monoxigenadas e dimetoxiladas nas mesmas posições.

Pelas similaridades estruturais entre a kielcorina **27** e a psorospermina (**18**) e com base nos estudos de citotoxicidade [18] e de inibição da topoisomerase II [73] obtidos para os xantonolignóides, foram avaliados pelo seu efeito no crescimento das linhas celulares tumorais humanas MCF-7, TK-10 e UACC-62, os análogos kielcorínicos sintetizados **29-33**, bem como a *trans*-(±)-kielcorina (**27**), a *trans*-(±)-kielcorina B (**34**) e a *trans*-(±)-isokielcorina B (**35**). Os resultados e as relações de estrutura-actividade para os xantonolignóides e para os seus precursores de síntese são apresentados no ***Anexo II*** (pág. 108-123)[*]. Comparando os resultados do ***Anexo II*** com os do ***Anexo V***, enquanto que as xantonas simples oxigenadas mostraram, em geral, uma maior capacidade de inibir o crescimento da linha celular UACC-62 (melanoma), os xantonolignóides revelaram-se mais activos para a MCF-7 (adenocarcinoma da mama).

Com o objectivo de interpretar os resultados obtidos no ***Anexo II*** para os xantonolignóides, foram calculadas as geometrias optimizadas para a psorospermina (**18**) e para a *trans*-kielcorina C (**29**) e determinados os potenciais electrostáticos (Fig. 3.1).

[*] Ensaios realizados no grupo de Bioensaios do CEQOFF-UP pela Drª Madalena Pedro sob orientação da Professora Doutora Maria de São José Nascimento.

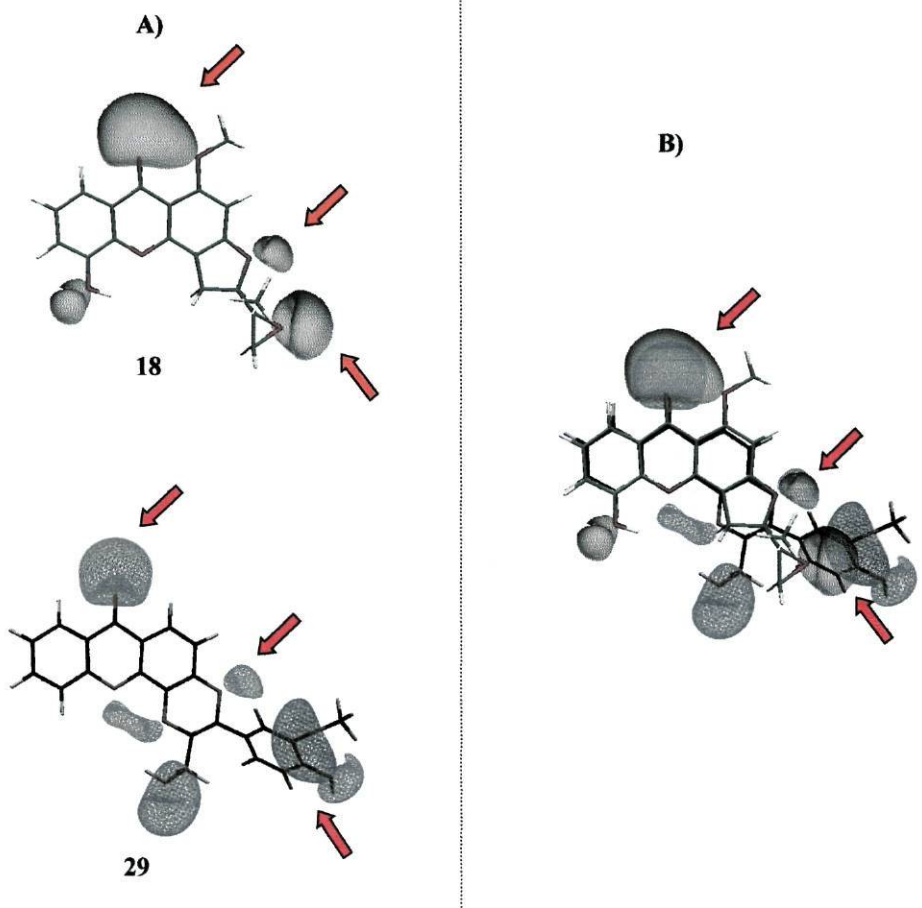


Fig. 3.1. Isosuperfícies de potencial a –20 kcal.mol^{-1}: **A)** para a psorospermina (**18**) e para a *trans*-kielcorina C (**29**). **B)** sobreposição da psorospermina (**18**) com a *trans*-kielcorina C (**29**).

Os compostos **18** e **29** possuem três locais idênticos de potencial negativo (assinalados na Fig. 3.1 com ⟹): um sobre o carbonilo do núcleo xantónico (**18**,**29**), um sobre o oxigénio do anel furano (**18**) e sobre o do dioxano (**29**) e um sobre o oxigénio do anel epóxido (**18**) e sobre os substituintes oxigenados do anel aromático do núcleo coniferilíco (**29**) (Fig. 3.1.**A**). A Fig. 3.1.**B** coloca em evidência o *fit* que proporciona uma maior coincidência estrutural e de superfícies de potenciais entre os pontos acima mencionados. Pela semelhança no carácter estereoelectrónico encontrado para estes compostos, poderá ser colocada a hipótese para a actividade dos xantonolignóides de um mecanismo relacionado com a intercalação no DNA e inibição da topoisomerase II.

Influência da Quiralidade dos Xantonolignóides

Tendo em consideração o efeito citotóxico da psorospermina (**18**) como enantioespecífico [94], foi efectuada a resolução, em escala semi-preparativa, da *trans*-(±)-kielcorina C (**29**), da *trans*-(±)-kielcorina D (**31**), da *trans*-(±)-isokielcorina D (**32**) e da *trans*-(±)-kielcorina E (**33**), apresentada no Capítulo 3. Procedeu-se à avaliação dos enantiómeros obtidos na inibição do crescimento celular na linha celular tumoral humana, MCF-7 (adenocarcinoma da mama) *, linha celular para a qual as misturas racêmicas **29**, **31-33** demonstraram maior potência.

A Tabela 3.3 coloca em evidência os resultados do efeito dos enantiómeros de **29**, **31-33** no crescimento da linha celular MCF-7, expresso em concentrações que provocam 50% de inibição do crescimento celular (GI_{50}).

Tabela 3.3. Efeito dos Enantiómeros das Misturas Racémicas **29**, **31-33** na Linha MCF-7

	Enantiómeros							
	29		**31**		**32**		**33**	
	(+)	(-)	(+)	(-)	(+)	(-)	(+)	(-)
GI_{50}[a] (μM)	39,4±2,2	7,6	29,9±2,8	48,5±2,9	55,0±5,8	34,9±1,9	19,8±1,7	22,9±1,0

[a] Os resultados são expressos em GI_{50} (concentrações do composto que provocam 50% de inibição do crescimento celular) como média ± erro padrão da média de 3-6 experiências independentes realizadas em duplicatas excepto para (-)-**29** que corresponde a 2 experiências independentes realizadas em duplicatas. A doxorrubicina foi usada como controlo positivo (GI_{50} MCF-7 = 42.8±8.2 nM).

Todos os enantiómeros testados inibiram o crescimento *in vitro* da linha MCF-7 tendo o enantiómero (-) da *trans*-(±)-kielcorina C (**29**) revelado uma potente actividade.

Por comparação entre os respectivos pares de enantiómeros:

- as actividades dos enantiómeros (-) da *trans*-kielcorina C (**29**) e da da *trans*-isokielcorina D (**32**) foram significativamente ($P<0,01$) maiores do que as dos enantiómeros (+);
- a actividade do enantiómero (+) da *trans*-kielcorina D (**31**) foi significativamente ($P<0,01$) maior do que a do enantiómero (-);

* Ensaios realizados no grupo de Bioensaios do CEQOFF-UP pela Drª Madalena Pedro sob orientação da Professora Doutora Maria de São José Nascimento.

- não se verificou uma diferença significativa ($P>0,1$) entre as actividades do par de enantiómeros da *trans*-kielcorina E (**33**).

Comparando os resultados obtidos para os enantiómeros com os resultados das misturas racémicas respectivas (***Anexo II***), foi possível verificar que:

- as actividades de ambos os enantiómeros (+) e (-) da *trans*-kielcorina D (**31**) e da *trans*-isokielcorina D (**32**) foram significativamente ($P<0,005$) superiores às actividades das misturas racémicas **31** ($GI_{50}>100$ μM) e **32** ($GI_{50}=82,8\pm4,3$μM), respectivamente;
- a actividade apresentada pelos enantiómeros (+) e (-) da *trans*-kielcorina E (**33**) não apresentou diferenças significativas ($P>0,1$) em relação à mistura racémica ($GI_{50}=21,2\pm8,2$μM);
- a actividade do enantiómero (-) da *trans*-kielcorina C (**29**) foi significativamente ($P<0,0001$) superior à actividade da mistura racémica **29** ($GI_{50}=18,3\pm0,7$μM), enquanto que o enantiómero (+) se mostrou significativamente ($P<0,0001$) menos activo que esta.

Pela similaridade estrutural e electrónica entre o composto **29** e a psorospermina (**18**) (Fig. 3.1), este resultado não será surpreendente se se considerar a importância da configuração do grupo epoxidi-hidrofurano para a actividade *in vivo* [94,95,101] e, sugere que a *trans*-(-)-kielcorina C poderá actuar por inibição da topoisomerase II.

Actividade Imunomoduladora

Muitos representantes de várias classes de compostos fenólicos revelaram ser moduladores de factores funcionais da resposta imune [102]. Concretamente, um conjunto de xantonas simples oxigenadas revelaram actividade anticomplementar [103-105] e várias xantonas hidroxigenadas demonstraram uma actividade anti-inflamatória [106-109]. Paralelamente, vários derivados foram descritos por interferir especificamente com a função de sistemas enzimáticos envolvidos no aparecimento do processo inflamatório, como a PKC [75-78], a esfingomielinase [62,63] e a fosfolipase C [35,72], enzimas intimamente envolvidas na tradução do sinal e no processo de activação celular envolvendo linfócitos T [110,111].

Investigações anteriores da actividade imunomoduladora de xantonas naturais

polioxigenadas efectuadas no CEQOFFUP, revelaram a 2-hidroxi-1-metoxixantona (**21**) como o composto com actividade mais marcada na inibição da proliferação de linfócitos T humanos[103]. Este resultado impulsionou a síntese de derivados 1- e/ou 2-oxigenados, apresentada no Capítulo 2.

No ***Anexo V*** (pág. 159-165) são descritos os resultados e algumas considerações sobre a relação da estrutura de vinte e sete xantonas oxigenadas e o seu o efeito na proliferação *in vitro* de linfócitos T humanos após estimulação com lectina mitogénica fito-hemaglutinina (PHA) [112*].

À semelhança de resultados anteriores que indicaram o grupo oxigenado em C(3) do esqueleto xantónico como um importante descritor, quer para a actividade anti-inflamatória[106] quer para efeitos antiplaquetários [113], neste estudo o mesmo padrão de substituição revelou ser importante para a actividade imunomoduladora.

Os resultados da avaliação do efeito dos xantonolignóides sintetizados, bem como da *trans*-(±)-kielcorina (**27**), da *trans*-(±)-kielcorina B (**34**) e da *trans*-(±)-isokielcorina B (**35**) sobre a proliferação de linfócitos T humanos são apresentados no ***Anexo II*** (pág. 108-123)*. Parecem ser factores de desfavorecimento para este efeito:

- a forma global "long-chair",
- a presença do metoxilo no núcleo xantónico na forma global "pistola",
- a isomeria 2'-hidroximetil-3'-aril na forma global "linear";

os xantonolignóides mostraram ser menos activos que os respectivos blocos contrutores xantónicos.

O mecanismo de acção pelo qual os derivados xantónicos estudados inibem a proliferação de linfócitos T não se encontra ainda elucidado. No que respeita os compostos fenólicos, a actividade imunomoduladora parece estar relacionada com a interacção com sistemas reguladores e produtores de radicais livres derivados de espécies de oxigénio [114]. As xantonas poderão interagir química e biologicamente com sistemas de transferência de electrões ou enzimas oxidantes ou redutoras. Pelo perfil demonstrado num estudo sobre a inibição da peroxidação lipídica e indução do factor de necrose tumoral por xantonas oxigenadas [115], foi proposto que di-hidroxixantonas que levassem à formação de estruturas quinóides estáveis seriam mais activas nas duas actividades investigadas. Analisando os resultados apresentados no ***Anexo V*** para as xantonas di-hidroxiladas é possível que a actividade antiproliferativa das xantonas possa estar relacionada com a formação de estruturas quinonóides.

* Ensaios realizados no grupo de Bioensaios do CEQOFF-UP pela Drª Fátima Cerqueira sob orientação da Professora Doutora Maria de São José Nascimento.

Outro mecanismo possível, compreende a inibição de enzimas reguladoras da divisão celular, na medida em que, várias xantonas foram já reconhecidas como inibidoras de várias quinases de proteínas como a PKC [75-78], a quinase de proteínas A [75,76] e a fosfolipase C [35,72], enzimas envolvidas na regulação de uma variedade de processos celulares como o metabolismo, a divisão celular e a expressão genética.

Pelo exposto, os resultados apresentados poderão se provenientes de mecanismos de acção distintos, como a ligação a proteínas, a interferências com locais activos enzimáticos ou devido a efeitos antioxidantes.

Modulação da Quinase C de Proteínas

A PKC constitui uma família de quinases de serina-treonina que cataliza numerosas reacções bioquímicas cruciais à função de muitos constituintes celulares com envolvimento na secrecção e exocitose, na modulação da condução iónica, na regulação da interacção do receptor com componentes do sistema da transdução do sinal, na contração do músculo liso, na expressão genética e na proliferação celular [9,11,116]. São exemplos desta última, a activação da topoisomerase II [117] ou da glicoproteína P [118], para as quais é requerida a fosforilação pela PKC.

A família da PKC incorpora várias isoformas que podem ser classificadas, com base

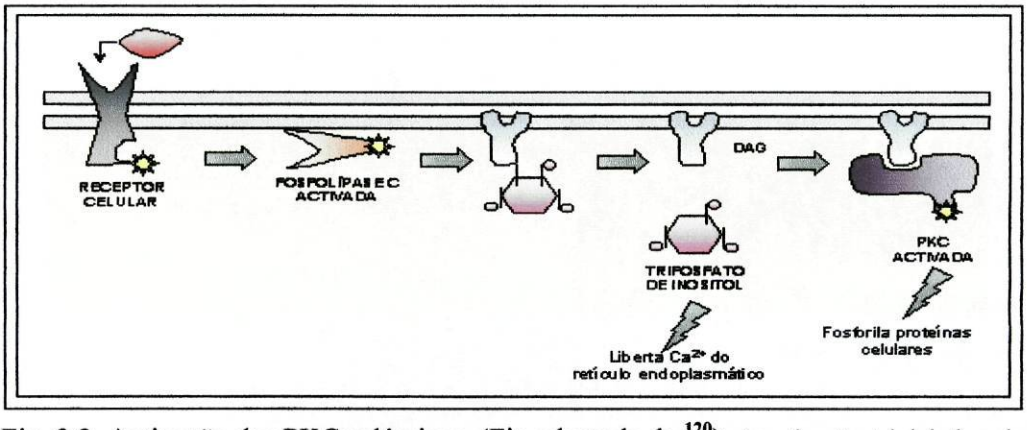


Fig. 3.2. Activação das PKCs clássicas. (Fig adaptada de [120]). A activação é iniciada pela interacção de hormonas, factores de crescimento ou mitogénios no receptor da superfície celular que conduzem à hidrólise, mediada pela fosfolipase C, de fosfolípidos de inositol em DAG e trifosfato de inositol; o DAG permite a activação da PKC a concentrações fisiológicas de cálcio [121,122]. A interacção da PKC com o DAG endógeno é estequiométrica e estereoespecífica, com o enantiómero (S) a ser o composto activo [121,123].

no seu modo de activação, nos quatro seguintes subgrupos [119]: o subgrupo das PKCs clássicas que inclui as isoformas α, βI, βII e γ, todas dependentes de cálcio, diacilglicerol (DAG) e fosfatidilserina (Fig. 3.2); o subgrupo das PKCs novas, que inclui as isoformas δ, ε, η e θ, independentes de cálcio, mas que requerem DAG e fosfatidilserina para a sua activação; o subgrupo das PKCs atípicas que inclui as isoformas ζ e ι/λ, cuja activação não requer cálcio e DAG, mas apenas fosfatidilserina; e o subgrupo das quinases PKC-relacionadas, que à semelhança das atípicas apenas necessita de fosfatidilserina para a sua activação.

A distribuição das diferentes isoformas da PKC revela uma especificidade considerável para certos tecidos e células e pode estar relacionada com o papel específico de cada isoforma na expressão genética durante o desenvolvimento, a homeostasia e a tumorogénese [11]. Algumas isoformas são expressas duma forma ubíqua, como por exemplo a PKCα, enquanto que outras são restritas a certos tecidos, como é o caso da PKCγ apenas detectada em tecidos neuronais, e das PKCη e PKCθ, maioritariamente na pele e músculo esquelético, respectivamente. Na Tabela 3.4 são apresentadas algumas das funções celulares associadas a isoformas da PKC.

Tabela 3.4. Funções Celulares Associadas às Isoformas da PKC Utilizadas Neste Trabalho

Isoformas da PKC	**Processos Associados**
PKCα	actividade aumentada no adenocarcinoma da mama e gliomas malignos e diminuída no carcinoma do cólon [124], comunicação intercelular e supressão da apoptose [125]
PKCβII	cardiomiopatia, falha cardíaca, complicações cardiovasculares diabete-induzidas [119] e cancro [125]
PKCδ	retardamento da progressão do ciclo celular na fase G_1 no músculo liso [126], supressão tumoral [119] e indução da diferenciação celular e da apoptose [125,127]
PKCη	diferenciação epitelial [125]
PKCζ	diferenciação e proliferação celular [126]

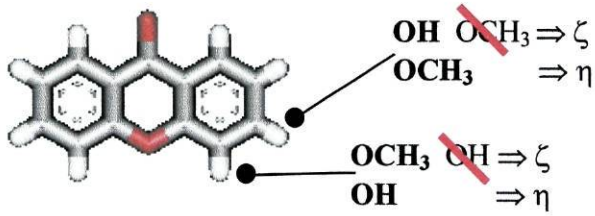
Para compreender melhor a função celular e fisiológica de cada isoforma da PKC é necessário o desenvolvimento de novos agentes farmacológicos que exibam selectividade para cada isoforma individualmente. A descoberta de que a PKC é um receptor com alta afinidade para os ésteres de forbol, promotores tumorais que activam a enzima por um mecanismo similar ao do DAG, estabeleceu a base do envolvimento da PKC na carcinogénese e concedeu uma ferramenta farmacológica poderosa para manipular a PKC, em sistemas *in vitro* e *in vivo* [120,128,129]. O efeito de vários ésteres de forbol na activação das PKCs α, βI, γ, δ e ε foi investigado, sem sucesso, na tentativa de identificar activadores de isoenzimas selectivos [130]. Apesar do problema da selectividade entre as isoformas desta importante família de quinases permanecer, a família da PKC continua a ser um alvo líder na descoberta de fármacos antineoplásicos [131].

No que diz respeito à classe das xantonas, várias xantonas preniladas [75,76] e duas xantonas simples oxigenadas [77,78] foram avaliadas na modulação na PKC. Enquanto que a 1,3,6,7-tetrahidroxixantona [77] e as xantonas preniladas [75,76], se mostraram inibidoras da PKC, a 1,7-di-hidroxixantona (**16**) foi capaz de mimitizar a indução da diferenciação celular de neuroblastomas, causada por activadores da PKC [78] e mostrou activar diferencialmente várias isoformas da PKC [132].

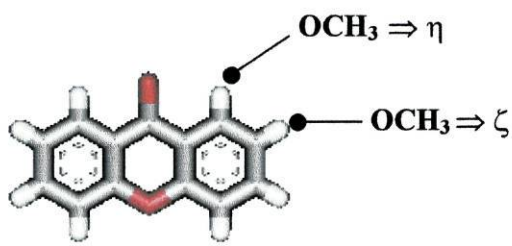
Pelo exposto, e com o intuito de procurar novos agentes quimioterápicos baseados na modulação da PKC e elucidar o mecanismo de acção dos derivados xantónicos na proliferação celular, foi estudado o efeito dos compostos sintetizados na modulação *in vitro* na PKC e *in vivo* nas isoformas α, βI, δ, η, e ζ da PKC. No ***Anexo VI*** * são apresentados os resultados obtidos para as xantonas oxigenadas simples que revelaram um efeito compatível com a activação da PKC, e no ***Anexo VII*** * para as que mostraram um efeito compatível com a inibição da PKC. O estudo da modulação pelos xantonolignóides nas várias isoformas é apresentado no ***Anexo VIII*** *.

O ***Anexo VI*** (pág. 166-175) expõe os resultados e aponta a relação estrutura-actividade para as xantonas oxigenadas simples que revelaram um efeito compatível com a activação da PKC. Relativamente à selectividade/potência apresentada pelos derivados xantónicos, podem acrescentar-se as seguintes relações para os substituintes do núcleo xantónico:

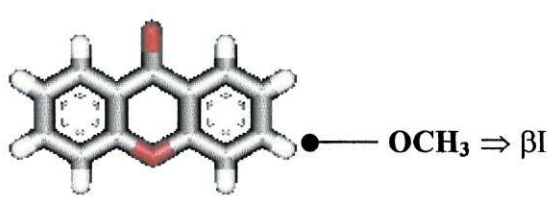
* Ensaios realizados no Laboratório de Farmacologia da Faculdade de Farmácia do Porto pela Drª Lucília Saraiva sob orientação do Professor Doutor Jorge Gonçalves, Professora Doutora Paula Fresco e Professora Doutora Eugénia Pinto.



A) um metoxilo em C(4) e/ou um hidroxilo em C(3) favorece(m) a interacção com a isoforma ζ, um metoxilo em C(3) e/ou um hidroxilo em C(4) na estrutura xantónica desfavorece(m) a interacção com a isoforma ζ, melhorando a interacção com a isoforma η (pela observação dos resultados para a 4-metoxi- (**8**), 3-hidroxi- (**7**) e 3-hidroxi-4-metoxixantona (**22**) em comparação com a 3-metoxi- (**6**), 4-hidroxi- (**9**) e 4-hidroxi-3-metoxixantona (**23**));



B) a substituição em C(1), particularmente por um metoxilo, confere maior potência para a isoforma η, a substituição em C(2), confere maior interacção para a isoforma ζ (pela observação dos resultados para a 1-metoxi- (**2**), 1-hidroxi- (**3**) e 1,2-dimetoxixantona (**10**) em comparação com a 2-hidroxi- (**5**), 2-metoxi- (**4**) e 1,2-di-hidroxixantona (**11**));

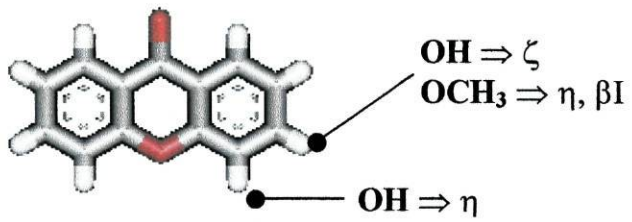


C) a presença do grupo 3-metoxi no esqueleto xantónico parece ser um descritor favorável na modulação da isoforma βI (pela observação dos resultados para a 3-metoxi- (**6**) e 2,3-dimetoxixantona (**12**)).

Apesar da escassez de dados cristalográficos para as isoenzimas da PKC, encontra-se proposto um farmacóforo comum aos activadores estruturalmente diversos da PKC [133]. Com base em estudos de relação estrutura-actividade por abordagens de modelização molecular [133], foram assumidas como características essenciais para a activação da PKCα, propriedades de hidrofobicidade adequadas e um padrão triangular entre átomos de oxigénio no farmacóforo proposto. No entanto, os resultados apresentados no ***Anexo VI*** parecem não se coadunar com este modelo: a xantona (**1**) não obedece ao requisito dos três pontos oxigenados e, não obstante, revelou um efeito compatível com a activação da PKCα. Este dado vem reforçar a hipótese apresentada neste trabalho, de que a ligação ao receptor pelas xantonas simples oxigenadas deve acontecer ou num local diferente ou por um mecanismo distinto do descrito para os activadores ésteres de forbol.

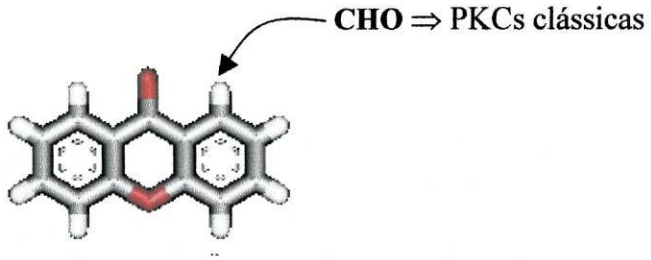
É assim de realçar o potencial das xantonas, não só como ferramentas na elucidação fisiológica das isoformas da PKC mas também na sua aplicação como agentes intervenientes no processo da apoptose e da carcinogénese de tecidos epiteliais (Tabela 3.4). Considerando os resultados obtidos no ***Anexo VI***, poderá ser proposta a aplicação da 4-hidroxi- (**9**) e da 2,3-dimetoxixantona (**12**) como activadores-padrão universais para estudos de modulação da PKC.

No ***Anexo VII*** (pág. 176-187) são apresentados os resultados para as xantonas simples oxigenadas que revelaram um efeito compatível com a inibição *in vitro* e *in vivo* da PKC, a 3,4-di-hidroxixantona (**15**) e a 1-formil-4-hidroxi-3-metoxixantona (**24**). Este trabalho vem reforçar a afirmação de que pequenas variações na estrutura do núcleo xantónico podem originar alterações drásticas na actividade/selectividade, em relação à modulação das diferentes isoformas da PKC (***Anexo VI***). Assim, por comparação com os resultados obtidos no ***Anexo VI*** para xantonas que mostraram activar a PKC, podem ser extraídas as seguintes relações entre a estrutura e a modulação das várias isoformas da PKC:



A) a manutenção de um hidroxilo em C(4) favorece a interacção selectiva com a isoforma η, como se verifica para a 3,4-di-hidroxixantona (**15**) e para os

activadores anteriormente apresentados; os resultados apresentados não contradizem o anteriormente estipulado, nem para o grupo hidroxilo em C(3), uma vez que, dos inibidores testados, a 3,4-di-hidroxixantona (**15**) mostrou ser o composto mais potente para a isoforma ζ, nem para o grupo metoxilo em C(3), uma vez que, a 1-formil-4- hidroxi-3-metoxixantona (**24**) interagiu com as isoformas βI e η;



B) a introdução de um grupo formilo em C(1) altera drasticamente o efeito na modulação da PKC, transformando o activador selectivo para a isoforma η da subfamília das PKCs novas, a 4-hidroxi-3-metoxixantona (**23**), num potente inibidor das PKCs clássicas, a 1-formil-4-hidroxi-3-metoxixantona (**24**).

Os resultados dos ***Anexos VI*** e ***VII*** para as xantonas simples oxigenadas, particularmente a inibição demonstrada pela 3,4-di-hidroxixantona (**15**), bloco construtor da *trans*-(±)-kielcorina C (**29**), e as semelhanças estereoelectrónicas encontradas para os xantonolignóides com a queleritrina conduziram à investigação na modulação da PKC por esta classe de compostos. No ***Anexo VIII*** (pág. 188-202) são descritos os resultados e a relação estrutura-actividade para os xantonolignóides e para o inibidor queleritrina na modulação *in vitro* da PKC e *in vivo* nas isoformas α, βI, δ, η, e ζ da PKC.

Da relação estrutura-actividade apresentada, pode sublinhar-se que as formas globais "pistola" e "linear" não favorecem a interacção com as isoformas α e δ. Comparando os resultados apresentados no ***Anexo VIII*** com os do ***Anexo VII***, é possível verificar que os xantonolignóides testados são, na generalidade, menos activos que os derivados simples oxigenados. No entanto, é curioso verificar que a *trans*-(±)-kielcorina C (**29**) mostrou ser o inibidor mais potente e selectivo para a isoforma ζ, enquanto que o seu bloco construtor xantónico, a 3,4-di-hidroxixantona (**15**), mostrou ser o inibidor mais potente e selectivo para a isoforma η.

Os resultados apresentados para os xantonolignóides e o conhecimento da preferência estereoquímica da PKC para (S)-diglicerídeos [123] e para o 4-β-isómero do 12-miristato-13-acetato de forbol [126], vieram reforçar o interesse na resolução das misturas racémicas dos xantonolignóides sintetizados (abordado no Capítulo 3).

Pelas similaridades biológicas e electrónicas encontradas, a ligação dos xantonolignóides à PKC poderá corresponder ao descrito para a queleritrina, isto é, poderá ocorrer no local de ligação da adenosina-trifosfato (ATP) da PKC [134]. Claramente, estudos experimentais terão que ser realizados para verificar esta proposta.

Nesta fase do conhecimento é difícil extrapolar para um todo as relações estruturais estabelecidas em cada actividade. Não obstante, a actividade antiproliferativa pode ser consequência da modulação na actividade da PKC celular, induzida por esta classe de compostos. A corroborar esta possibilidade parecem estar os resultados obtidos para a 3,4-di-hidroxixantona (**15**) na medida em que revelou selectividade significativa, quer para a linha celular de melanoma (UACC-62), quer para a PKCη, isoforma associada à diferenciação do tecido epitelial. É relevante o facto dos compostos estudados terem demonstrado modular diferencialmente as várias isoformas da PKC; um melhor entendimento de como as isoenzimas individuais contribuem para a patogénese do cancro permitirá, certamente, o planeamento de compostos em que estas isoenzimas se destaquem como alvos terapêuticos viáveis [135].

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Capítulo 5

Conclusões

Conclusões

As xantonas e os xantonolignóides são alvos estruturais interessantes na procura de compostos farmacologicamente activos. Compostos com o núcleo xantónico apresentam um leque notável de propriedades bioquímicas e farmacológicas, algumas já relacionadas com vários alvos celulares.

A síntese de xantonas e xantonolignóides planeada foi realizada com sucesso. O método de síntese de condensação de um derivado do ácido salicílico com um fenol apropriado pela reacção de Ullmann, seguida da ciclização regioespecífica com LDA, permitiu obter, em poucas etapas e com um alto rendimento, xantonas 1,2-dioxigenadas. Na síntese biomimética de xantonolignóides, o método mais eficiente envolveu o uso de $K_3[Fe(CN)_6]$, como o agente oxidante; no entanto, nenhum dos agentes oxidantes utilizados, Ag_2O, Ag_2CO_3 e $K_3[Fe(CN)_6]$, conferiu regio- ou estereosselectividade à reacção de acoplamento oxidativo. A *cis*-(±)-kielcorina C (**30**) foi convertida eficazmente no respectivo *trans*-isómero **29**, por tratamento com K_2CO_3/H_2O. No processo biomimético de síntese de xantonolignóides, os resultados experimentais observados mostraram que a posição dos substituintes hidroxilo tem um efeito determinante na natureza dos regioisómeros kielcorínicos obtidos: a influência da distribuição electrónica no percursor xantónico *orto*-di-hidroxilado permite prever o regioisómero mais abundante formado. O comportamento regioquímico da reacção foi justificado por estudos computacionais. Os dados apresentados suportam o mecanismo proposto para a síntese biomimética de xantonolignóides. A metodologia de HPLC desenvolvida para a separação e quantificação nos produtos brutos da reacção provou ser um método eficiente para a análise destes derivados xantónicos. Técnicas de INEPT selectivo, NOE e HETCOR permitiram determinar e/ou corrigir elucidações estruturais dos derivados xantónicos obtidos.

No método descrito para a separação de enantiómeros das kielcorinas, as fases estacionárias de carbamatos derivados de polissacarídeos de amilose mostraram maior poder de discriminação quiral que as de celulose. A eluição em fases móveis do tipo polar-orgânico mostrou vantagens na separação deste tipo de compostos. A coluna de *tris*-3,5-dimetilfenilcarbamato amilose foi a selecionada para a separação semi-preparativa das misturas racémicas *trans*-(±)-kielcorina C (**29**), *trans*-(±)-kielcorina D (**31**), *trans*-(±)-isokielcorina D (**32**) e *trans*-(±)-kielcorina E (**33**).

Dos xantonolignóides sintetizados, a *trans*-(±)-kielcorina C (**29**) e a *trans*-(±)-kielcorina E (**33**) mostraram um efeito superior na inibição do crescimento de linhas celulares tumorais humanas. A estereoquímica nos xantonolignóides mostrou influenciar o efeito citotóxico, tendo o enantiómero dextrógiro da mistura racémica **29** revelado uma potente inibição no crescimento celular na linha MCF-7 (adenocarcinoma da mama). Das xantonas simples oxigenadas, a 1,2- (**11**) e 3,4-di-hidroxixantona (**15**) revelaram uma selectividade significativa para a linha UACC-62 (melanoma) e a di-hidroxilação no núcleo xantónico foi associada a uma actividade inibitória superior. Os derivados xantónicos simples oxigenados mostraram-se mais potentes que os xantonolignóides na inibição da proliferação de linfócitos T e a 3,4-di-hidroxixantona (**15**) exibiu a maior actividade. Das relações estrutura-actividade apresentadas para estas actividades biológicas, propõem-se mecanismos de acção distintos para os xantonolignóides e para as xantonas.

Da série de xantonas simples oxigenadas avaliadas pelo seu efeito na modulação da PKC, um grupo de dezasseis compostos causou um efeito compatível com a activação da PKC, duas xantonas causaram um efeito compatível com a inibição da PKC, e um grupo de quatro xantonas foi ineficaz na modulação de qualquer isoforma da PKC. Com a excepção para a 3,4-di-hidroxixantona (**15**), que revelou um efeito compatível com a inibição da PKC e selectividade para a isoforma η, a grande maioria das xantonas simples oxigenadas causou um efeito compatível com a activação da PKC e algumas mostraram alta selectividade para as isoformas δ, η ou ζ. Todos os xantonolignóides revelaram um efeito compatível com a inibição da PKC, similar ao do reconhecido inibidor queleritrina, com a *trans*-(±)-kielcorina C (**29**) a mostrar elevada selectividade para a isoforma ζ. Conclui-se que pequenas variações na estrutura do núcleo xantónico, originam alterações drásticas na actividade/selectividade em relação à modulação das diferentes isoformas da PKC. O estudo apresentado consiste na primeira abordagem para esclarecer o mecanismo molecular da actividade biológica da família dos xantonolignóides. Por outro lado, a elevada potência e selectividade demonstrada para as diferentes isoformas da PKC, sugere que os derivados xantónicos podem ser um grupo importante na procura de moduladores potentes e selectivos da PKC.

Os estudos incluídos nesta dissertação contribuíram fundamentalmente para:

- o aprofundamento do conhecimento a nível químico e biológico do grupo dos xantonolignóides, cujo papel era ainda pouco compreendido;
- a evolução no conhecimento de actividades biológicas de xantonas oxigenadas simples;

- a obtenção de potenciais líderes com actividade antitumoral, nomeadamente a 3,4-di-hidroxixantona (**15**) e a *trans*-(-)-kielcorina C.

Espera-se que estes estudos constituam uma plataforma para o futuro desenvolvimento de fármacos de natureza xantónica. No entanto, os fármacos são muito mais do que ligandos com elevada afinidade para os alvos. São compostos de alta tecnologia que requerem um balanço preciso entre a potência, selectividade, distribuição-metabolismo, e propriedades farmacocinéticas, de modo a possuírem um comportamento seguro no organismo humano.

Quando cremos que temos todas as respostas, de repente mudam todas as perguntas.

Mario Benetti

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ANEXOS

ANEXO I

Natural and Synthetic Xanthonolignoids: Chemistry and Biological Activities

M.M.M. Pinto* and E.P. Sousa

Centro de Estudos de Química Orgânica, Fitoquímica e Farmacologia da Universidade do Porto, Faculdade de Farmácia, Rua Aníbal Cunha, 164, 4050-047, Porto, Portugal



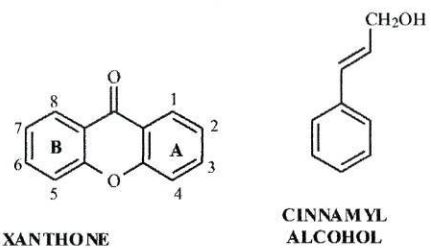
Abstract: Natural xanthonolignoids are formed by the coupling of cinnamyl alcohol with an appropriate *ortho*-dihydroxyxanthone. More than twenty xanthonolignoids have been isolated and synthesized and some interesting pharmacological activities of these compounds have been described.

The purpose of this review is to describe the natural occurrence, isolation, structure elucidation and synthesis – both biomimetic and classic –as well as biological activities of xanthonolignoids. Aspects related to biosynthesis will also be considered.

Key words: xanthonolignoid, xanthone, 1,4-benzodioxane, oxidative coupling, Clusiaceae

INTRODUCTION

Natural xanthonolignoids are a class of compounds with a phenylpropane (C_6-C_3 unit) skeleton linked to an *ortho*-dihydroxyxanthone by a dioxane ring, formed by radical oxidative coupling [1-3]. The building blocks of xanthonolignoids are simple *ortho*-dioxygenated xanthones and a C_6-C_3 unit represented by cinnamyl alcohol (Fig. (**1**)).



XANTHONE CINNAMYL ALCOHOL

Fig. (1).

Although information regarding xanthonolignoids can be found dispersed in some reviews on natural xanthones [4-9] and lignoids [4,10], no data considering these compounds as a separated group has yet been published.

Xanthonolignoids can be divided into the frameworks I-VI according to the location of the dioxane ring in the xanthone skeleton (Fig. (**2**)).

Table 1 shows natural xanthonolignoids which include the subgroups kielcorins, cadensins, subalatin, calophyllumins and gemixanthone, all of which having a framework corresponding to I, II, and III. While compounds of the framework I-III are natural products and have also been obtained either by classic or biomimetic synthesis, the compounds of frameworks IV, V, and VI have only been reported by synthesis.

The main challenge in the structure elucidation of xanthonolignoids was the definition of the regioisomerism for the phenyl and hydroxymethyl substituents on the 1,4-dioxane ring. The difficulties in determining the structures of such compounds are clearly demonstrated by the history of kielcorin, which is the first representative of this class of compounds [11-14]. The configuration and the regioisomerism of xanthonolignoids were initially established by chemical methods including derivatization [13,15-19], cleavage [1,14,15,17,21], and total synthesis [22,23], and further, by spectral analysis [1,2,13,14,16,17,20,21,24]. The unequivocal structure elucidation of xanthonolignoids has been ultimately achieved by a combination of 1D and 2D NMR techniques such as NOE, selective INEPT, COSY, HETCOR, HSQC, and HMBC [18,19,22,25-27].

The xanthonolignoids can be considered to be very promising compounds due to a large variety of interesting biological activities associated with xanthones [28-38] and the benzodioxane moiety [39-48]. In spite of this, only few data are available for xanthonolignoids [17,27,28,49].

NATURAL OCCURRENCE AND ISOLATION

Xanthonolignoids have been isolated from the plants of Clusiaceae family (Guttiferae) [21] especially from the genera *Cratoxylum* [25], *Kielmeyera* [20], *Caraipa* [13], *Hypericum* [14], *Psorospermum* [17], *Vismia* [16], *Harungana* [19], and *Calophyllum* [19]. As a result, they are considered as chemotaxonomically useful compounds [1,3,21].

*Address correspondence to this author at the Centro de Estudos de Química Orgânica, Fitoquímica e Farmacologia da Universidade do Porto, Faculdade de Farmácia, Rua Aníbal Cunha, 164, 4050-047, Porto, Portugal; Tel: /fax:+351-222057358; E-mail: madalena@ff.up.pt

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Table 1. Naturally Occurring Xanthonolignoids

Compounds	References
Kielcorin (1)	*Kielmeyera coriacea* Mart. [11,13,50]
(I) R_1=R_2=R_4=R_7=H	*K. corymbosa* Mart [11]
R_3=R_5=OMe	*K. speciosa* St. Hill [11]
R_6=OH	*K. ferriginea* A.P. Duarte [11]
	K. rubiflora Camb. [12]
	Hypericum androsaemum L. [14,51]
	H. calycinum L. [14]
	H. maculatum Cratz. [14]
	H. perforatum L. [14]
	H. ericoides L. [52]
	H. canariensis L. [1]
	H. mysorense Heyne [22]
	H. subalatum Hayata [21]
	H. reflexum [2]
	Vismia guaranirangae Huber [16]
	Psorospermum febrifugum Sprach [17]
Cadensin A (2)	*Carapaia grandiflora* Mart. [53]
(I/II) R_1=R_7=OH	*C. valoi* Paula [53]
R_2=R_4=R_5=H	*C. densiflora* Mart. [13]
R_3=R_6=OMe	*Vismia guaranirangae* Hubber [16]
	Hypericum henryi [3]
Cadensin B (3)	*Carapaia densiflora* Mart. [13]
(I/II) R_1=R_7=OH	*Vismia guaranirangae* Huber [16]
R_2=R_5=R_6=OMe	
R_2=R_5=H	
Cadensin C (4)	*H. canariensis* L [6]
(I) R_1=R_6=OH	*Vismia guaranirangae* Huber [16]
R_2=R_4=H	*Harungana madagascarensis* Lam ex Poir [19]
R_3=R_5=R_7=OMe	
Cadensin D or Hipericorin (5)	*Hypericum canariensis* L. [1]
(I) R_1=R_2=R_4=H	*H. mysorense* Heyne [15]
R_3=R_5=R_7=OMe R_6=OH	*H. subalatum* Hayata [21]
	Psorospermum febrifugum Sprach [17]
Kielcorin B (6)	*Kielmeyera coriacea* Mart. [20]
(III) R_1=R_2=R_4=R_7=H	
R_3=R_5=OMe	
R_6=OH	
Subalatin (7)	*Hypericum subalatum* Hayata [21]
(I) R_1=R_2=R_4=H	
R_3=R_6=OH	
R_5=R_7=OMe	

(Table 1). contd...

Compounds	References
Isocadensin D (8) (I) R_1=R_3=R_5=OMe R_2=R_4=R_7=H R_6=OH	*Psorospermum febrifugum* Sprach [17]
Isocadensin D monoacetate (9) (I) R_1=R_3=R_5=OMe R_2=R_4=R_7=H R_6=OAc	*Psorospermum febrifugum* Sprach [17]
Cadensin F (10) (I) R_1=R_3=R_5=R_7=OMe R_2=R_4=H R_6=OH	*Psorospermum febrifugum* Sprach [17]
6-Hydroxycadensin F(11) (I) R_1=R_3=R_5=R_7=OMe R_2=R_6=OH R_4=H	*Psorospermum febrifugum* Sprach [17] *Calophyllum inophyllum* L. [19]
Cadensin G(12) (I) R_1=R_2=R_6=OH R_3=R_4=H R_5=R_7=OH	*Psorospermum febrifugum* Sprach [17]
6-Methoxykielcorin (13) (I) R_1=R_4=R_7=H R_2=R_3=R_5=OMe R_6=OMe	*Hypericum reflexum* [2]
5'-Demethoxycadensin G (14) (I) R_1=R_2=R_6=OH R_3=R_4=R_7=H R_5=OMe	*Cratoxylum cochinchinense* (Lour.) B1 [25]
Calophyllumin A (15) (I) R_1=R_2=R_6=OH R_3=R_5=R_7=OMe R_4=H	*Calophyllum inophyllum* L. [19]
Calophyllumin B (16) (I) R_1=R_3=R_5=OMe R_2=R_6=OH R_4=R_7=H	*Calophyllum austroindicum* Kosterm ex P. F. Stevens [19]
Gemixanthone A (17) (II) R_1=R_3=R_5=R_7=OMe R_2=R_6=OH R_4=H	*Hypericum geminiflorum* [18]

Seventeen natural xanthonolignoids have been isolated so far. The structural frameworks and their isolation from natural sources are indicated, by chronological order in Table 1 and Fig. (**2**).

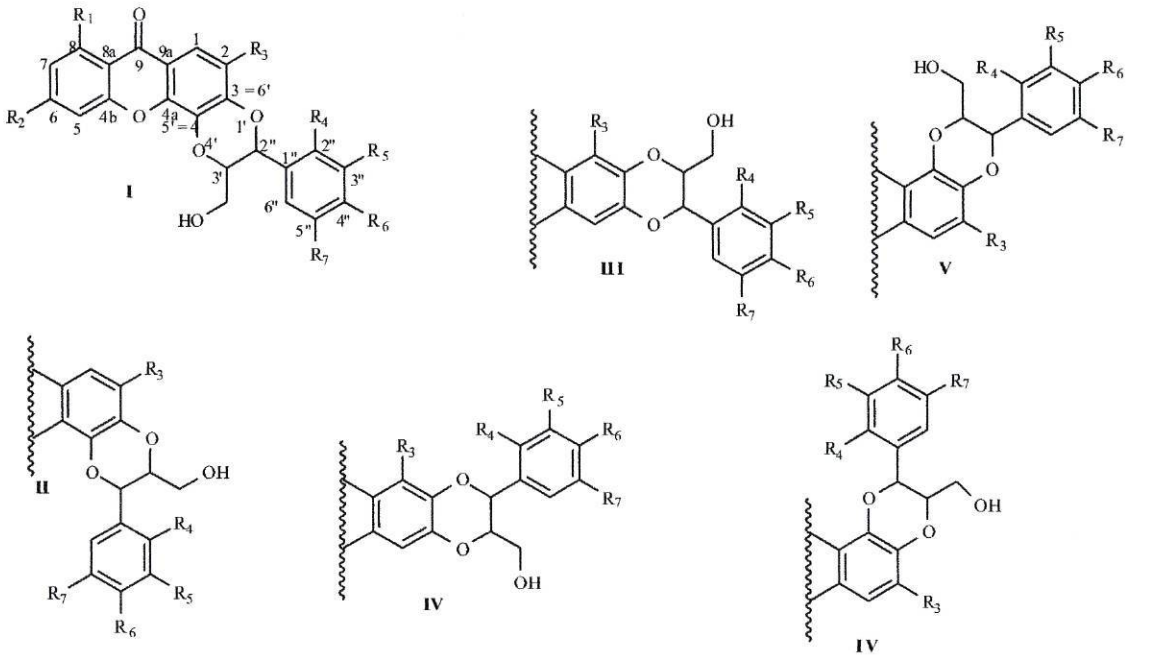


Fig. (2). Frameworks for natural/synthetic xanthonolignoids*.
*The frameworks are numbered in accordance with IUPAC rules.

The compounds isolated can be grouped as follows:

Kielcorins

The first compound of the xanthonolignoids group was described as a 2,3,4-trioxygenated xanthone isolated in 1969 from *Kielmeyera* species (Guttiferae) in a short communication by Gottlieb *et col.* [11]. Two years later, the compound named "kielcorin" was reported to be isolated from *Kielmeyera rubriflora* [12]. Two structures (Fig. (**3**). a, b) for the *trans*–isomer were then proposed for kielcorin [13].

By a chemical method involving basic hydrolysis of the benzyl ether linkage, Nielsen and Arends in 1978 [14] considered kielcorin to be a mixture of the enantiomers of (a) (Fig. (**3**)). Later, kielcorin (**1**) was also isolated from *Hypericum* species [1,2,15,21,22,51,52], *Vismia guaramirangae* [16], and *Psorospermum febrifugum* [17]. Another compound called kielcorin B (**6**) was isolated in a very low yield from *Kielmeyera* species [11] and its structure was tentatively assigned [20], being the only natural kielcorin with a skeleton of type III. In 1990, a new compound with an additional methoxy group on C-6 of the xanthone moiety was isolated from *Hypericum reflexum* [2] and was named 6-methoxykielcorin (**13**).

Cadensins

So far, ten xanthonolignoids have been reported as having the cadensin structural type. These are cadensins A (**2**), B (**3**), C (**4**), D (**5**), isoD (**8**), isoD monoacetate (**9**), F (**10**), 6-hydroxyF (**11**), G (**12**), and 5'-demethoxyG (**14**) [1,3,13,16,17,19,21,25,53]. The first compounds to be isolated in this class were cadensins A (**2**) and B (**3**) from

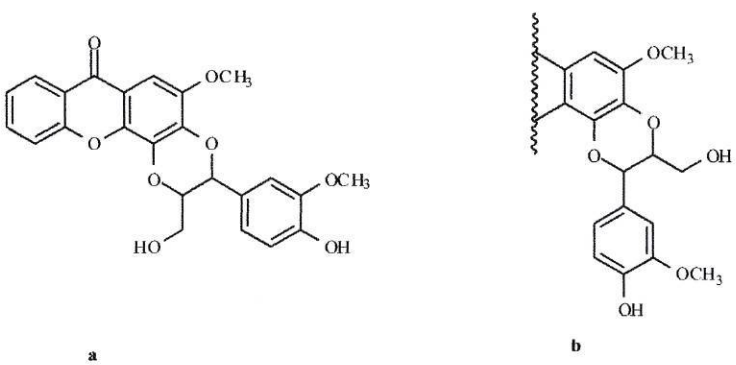


Fig. (3).

Caraipa densiflora [13]. Interestingly, in 1986 the same structure (**5**) was assigned to both cadensin D and hypericorin, isolated from *Hypericum canariensis* [1] and *H. mysorense* [15] respectively.

Subalatin

Actually, subalatin itself (**7**) is the only representative of this group [21]. Interestingly, subalatin is the only xanthonolignoid with a hydroxy instead of a methoxy group, on C-2 of the xanthone nucleus.

Gemixanthone

Gemixanthone (**17**) has been isolated from *Hypericum geminiflorum* [18] and for the first time, HMBC and NOESY NMR techniques were used for structure determination. Structurally, gemixanthone (**17**) and 6-hydroxycadensin F (**11**) [17] are positional isomers. However, while 6-hydroxycadensin F (**11**) has its structure belonging to the framework I, the structure of gemixanthone (**17**) contains the framework II.

Calophyllumins

Calophyllumins A (**15**) and B (**16**) are xanthonolignoids having a skeleton based on framework I and owe their names to *Calophyllum inophyllum*, the plant material from which they were isolated [19].

The natural xanthonolignoids referred in Table 1 (**1-17**) are structurally very similar [7]. In the majority of these compounds (**1-5, 7-17**), the dioxane bridge is located at positions 3,4 of the xanthone nucleus (I/II), except for kielcorin B (**6**), whose xanthonic nucleus containing *ortho*-dihydroxy groups on C-2 and C-3 (III) (Fig. (**2**)).

The oxygenation patterns of xanthonolignoids are found at Table 2.

All the natural xanthonolignoids show a *trans* configuration for the benzodioxane substituents and $[\alpha]^{20}_{D}=0$ except for gemixanthone A (-21°) [18] and subalatin (+600°) [21]. For the compounds **8-12** and **14**, the optical rotation was not indicated [17,25].

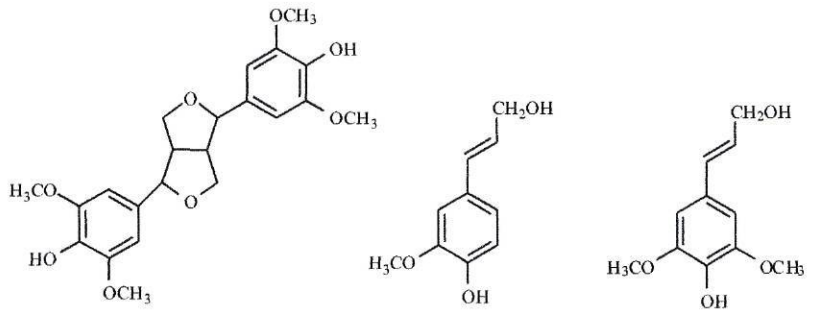
BIOSYNTHESIS OF XANTHONOLIGNOIDS

The biogenetic origin of the xanthonolignoids involves a dioxane ring formation between the xanthone moiety and cinnamyl alcohol derivative. Several studies have shown that oxygenated xanthones were derived from acetate-shikimate pathway [54-56]. The carbons of the acetate-malonate derived ring A are assigned 1-4 while the carbons of the shikimate derived ring B are numbered 5-8. This numbering is for biogenetic purposes only. Furthermore, the 6- and/or 8-oxygenation pattern in the xanthone moiety of the compounds **2-4** and **8-17** suggests that their dioxane ring is on the shikimate derived ring B. The formation of the benzodioxane ring can be generally postulated via radical oxidative coupling of an *ortho*-dihydroxyxanthone and cinnamyl alcohol [1-3,6] similar to those proposed for neolignans [57], flavanolignoids [58], and coumarinolignoids [59] respectively. This hypothesis is supported by the following co-occurrence data:

- Kielcorin (**1**) has been found together with 2,3,4-trioxygenated xanthones in three *Kielmeyera* species [6,50] as well as in *Hypericum calycinum*, in *H. ericoides* [6], and in *H. reflexum* [2];
- 6-Methoxykielcorin (**13**) and 2,3,4,6-tetraoxygenated xanthones were both isolated from *H. reflexum* [2];
- Cadensins A (**2**) and C (**4**) have been found together with xanthones with the corresponding oxygenation pattern in *Caraipa* species and *H. canariens* [6] respectively;
- 5'-Demethoxycadensin G (**14**) has been found to co-occur with 1,3,5,6-tetrahydroxyxanthone in *Cratoxylum cochinchinense* [25];
- The isolation of a syringaresinol (**18**) from *Vismia quaramirangae* [6] and *Vismia guaramirangae* [16] as a precursor of the C_6-C_3 unit in cadensins A (**2**), B (**3**),

Table 2. Oxygenation Patterns of Xanthonolignoids

Pattern of substitution			
Xanthone moiety		Phenylpropane moiety	
Oxygenated positions	Compounds	Oxygenated Positions	Compounds
2,3,4-	**1,5,7**	3",4"-	**1-2, 6, 8-9, 13-14, 16**
1,2,3-	**6**		
2,3,4,8,-	**2-4, 8-10**	3",4",5"	**4-5, 7, 10-12, 15, 17**
3,4,6,8-	**12, 14**		
2,3,4,6-	**13**	2",3",4"	**3**
2,3,4,6,8,-	**11, 15-17**		



18 20 21

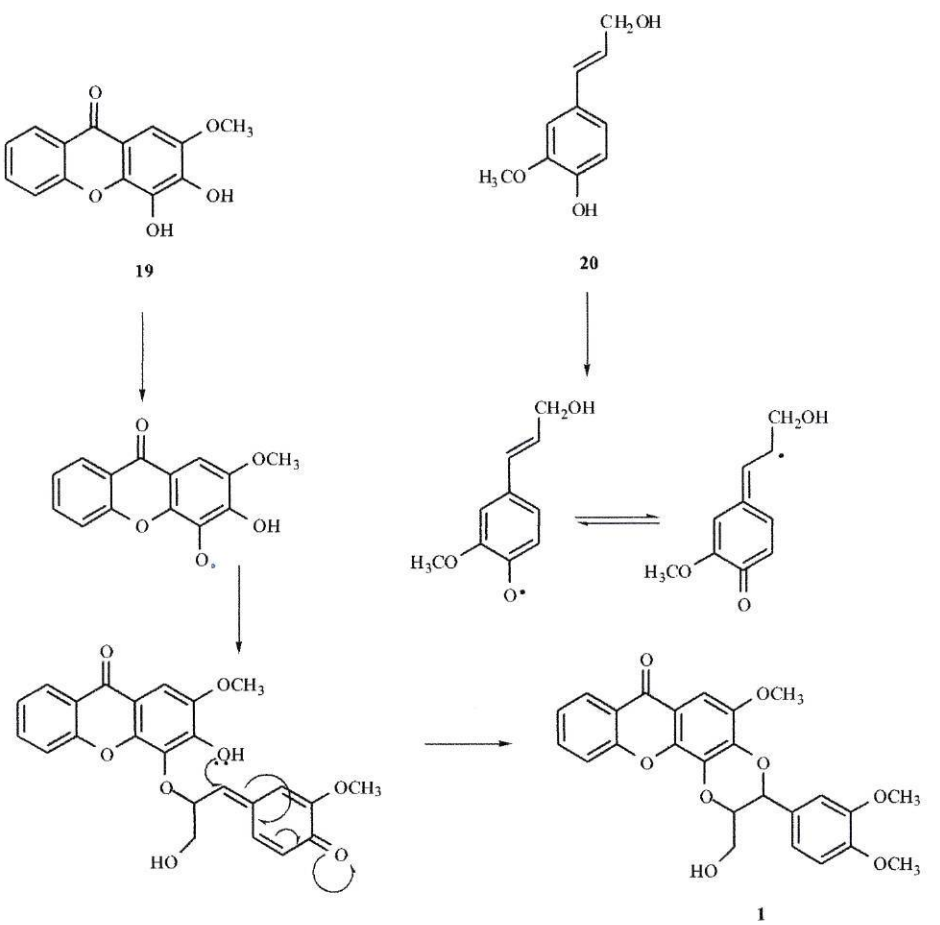
Fig. (4).

and C (**4**) could substantiate the above mentioned hypothesis.

Cardona *et al.* [1] have proposed the biosynthetic pathway for kielcorin (**1**) and cadensin D (**5**) as a result of a radical oxidative coupling of 3,4-dihydroxy-2-methoxyxanthone (**19**) with coniferyl alcohol (**20**) and sinapyl alcohol (**21**) respectively.

The formation of the free radical intermediates **20a** and **20b** of coniferyl alcohol (**20**) by oxidase in lignification process of developing xylems [60] is further strong evidence to support the oxidative coupling hypothesis for the benzodioxane ring formation in xanthonolignoids. The biosynthetic pathway for kielcorin (**1**) is proposed in Fig. (**5**).

Xanthonolignoids are also considered as useful chemotaxonomic markers [1-3,6,21] since they are isolated only from the plants of Clusiaceae (Guttiferae) family. The isolation of cadensins A (**2**) [3] and D (**5**) [1,15,21], of subalatin (**7**) [21], and of gemixanthone (**17**) [18], from



19 20 1

Fig. (5).

Hypericum species, was used to establish a link between the subfamilies Kielmeyeroideae and Hypericoideae. Furthermore, the occurrence of kielcorins **1** and **13** in *H. reflexum* [2] has supported its inclusion in the Clusiaceae family.

SYNTHESIS OF XANTHONOLIGNOIDS

Table 3 and Fig. (**2**) show the xanthonolignoids obtained by synthesis, in chronological order. The main goal of xanthonolignoid synthesis was initially for structure elucidation. Due to potential biological and pharmacological activities, it was necessary to obtain larger quantities of the compounds for bioassays. As a result, synthetic approaches have been directed mainly towards kielcorin derivatives [22,26-28].

Both total synthesis and one step biomimetic approaches have been used to obtain xanthonolignoids. In the classic synthesis, several steps are required to obtain the 1,4-dioxane moiety. Through the condensation of a benzyloxy-

Table 3. Xanthonolignoids Obtained by Synthesis

Compounds	Reference
Kielcorin (1) (**I**) R_1=R_2=R_4=R_7=H R_3=R_5=OMe R_6=OH	Classic [22,24] Biomimetic [20]
Demethoxykielcorin or *trans*-(±)-3'-hydroxymethyl-2'-(4"-hydroxy-3"-methoxyphenyl)-1',4'-dioxane[5',6'-*c*]xanthone (**22**) (**I**) R_1=R_2=R_3=R_4=R_7=H R_5=OMe R_6=OH	Classic [22] Biomimetic [27]
***cis*-Kielcorin (25)** (**I**) R_1=R_2=R_4=R_7=H R_3=R_5=OMe R_6=OH	Biomimetic [20] Classic [24]
5'-Demethoxycadensin G(14) (**I**) R_1=R_2=R_6=OH R_3=R_4=R_7=H R_5=OMe	Biomimetic [23]
***trans*-Kielcorin B (6)** (**III**) R_1=R_2=R_4=R_7=H R_3=R_5=OMe R_6=OH	Biomimetic [26]
***trans*-Isokielcorin B (26)** (**IV**) R_1=R_2=R_4=R_7=H R_3=R_5=OMe R_6=OH	Biomimetic [26]
cis—(±)-3'-Hydroxymethyl-2'-(4"-hydroxy-3"-methoxyphenyl)-1',4'-dioxane[5',6'-*c*]xanthone (**27**) (**I**) R_1=R_2=R_3=R_4=R_7=H R_5=OMe R_6=OH	Biomimetic [27]
trans-(±)-2'-Hydroxymethyl-3'-(4"-hydroxy-3"-methoxyphenyl)-1',4'-dioxane[5',6'-*a*]xanthone (**28**) (**V**) R_1=R_2=R_3=R_4=R_7=H R_5=OMe R_6=OH	Biomimetic [27]
trans-(±)-3'-Hydroxymethyl-2'-(4"-hydroxy-3"-methoxyphenyl)-1'4'-dioxane[5',6'-*a*]xanthone (**29**) (**VI**) R_1=R_2=R_3=R_4=R_7=H R_5=OMe R_6=OH	Biomimetic [27]
trans-(±)-2'-Hydroxymethyl-3'-(4"-hydroxy-3"-methoxyphenyl)-1',4'-dioxane[5',6'-*b*]xanthone (**30**) (**III**) R_1=R_2=R_3=R_4=R_7=H R_5=OMe R_6=OH	Biomimetic [27]

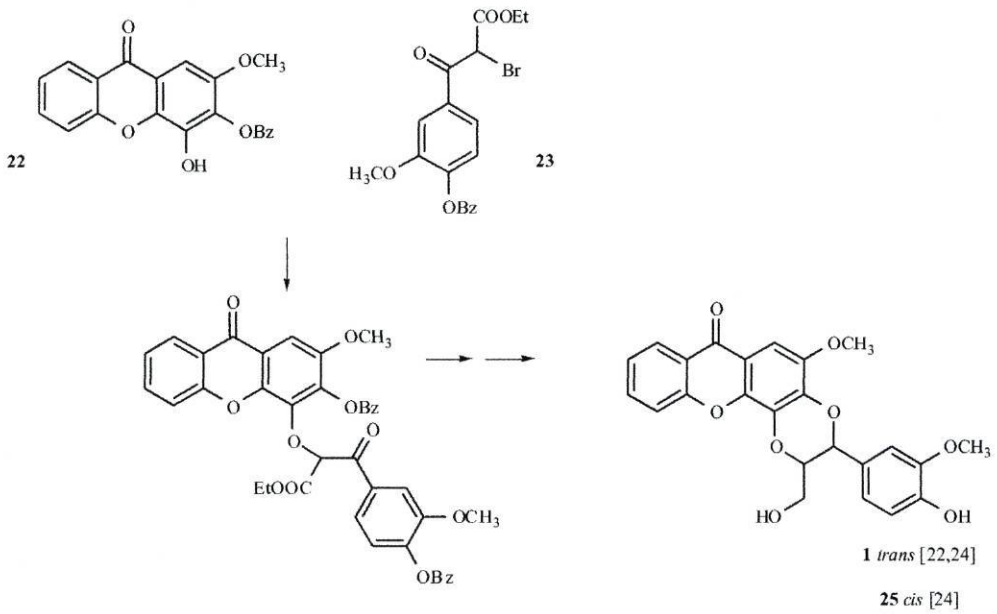


Fig. (6).

hydroxyxanthone (**22**) and ethyl 2-bromo-3-(4-benzyloxy-3-methoxyphenyl)-3-oxopropionate (**23**) with further cyclization, kielcorin (**1**) was obtained (Fig. (**6**)) [22,24].

Besides kielcorin (**1**), demethoxykielcorin (**24**) was also synthesized [22]. Although Tanaka *et al.* have used a similar route, a mixture of kielcorin (**1**) and its stereoisomer, *cis*-kielcorin (**25**), were obtained [24].

The biomimetic type synthesis of xanthonolignoids is based on natural building blocks and is achieved by an oxidative coupling of a suitable dihydroxyxanthone and a cinnamyl alcohol derivative, in the presence of an oxidizing agent such as silver oxide, silver carbonate or potassium hexacyanoferrate (III) at room temperature. Stereo- and/or regioisomers are generally formed [20,23,26,27]. The structure 5'-demethoxycadensin G (**14**), a xanthonolignoid isolated from *Cratoxylum cochinchinense* [25] in very low quantity, was unequivocally assigned only after the biomimetic synthesis. Selective INEPT experiments were used for the first time to elucidate its structure as well as to establish the orientation of the substituents on the 1,4-dioxane nucleus [23]. Pinto *et al.* [20] have reported the biomimetic synthesis of *trans*-kielcorin (**1**) and a minor compound *cis*-kielcorin (**25**) by oxidative coupling of 2-methoxy-3,4-dihydroxyxanthone (**19**) and coniferyl alcohol (**20**) in the presence of silver oxide (Fig. (**7**)). This was the first biomimetic synthesis of xanthonolignoids of the kielcorin group.

The mechanism proposed for this one-step approach is based on the oxidative coupling equivalent to that found in its biogenetic pathway (Fig. (**5**)). In a similar fashion, two other xanthonolignoids, *trans*-kielcorin B (**6**) and *trans*-isokielcorin B (**26**), were obtained and their structures were established by 2D NMR techniques [26].

Many members of the Clusiaceae family are receiving growing attention owing to their biological importance as well as their chemical diversity [6,7,9]. Table 4 gives the xanthonolignoids containing species used in traditional medicine.

Due to a variety of biological and pharmacological activities demonstrated by the xanthonolignoid containing species, the synthesis of several new kielcorin analogues were designed and developed in our group. Consequently, the following isomers were synthesized: *trans* (**24**) and *cis* (**27**) isomers of (±)-3'-hydroxymethyl-2'-(4''-hydroxy-3''-methoxyphenyl)-1',4'-dioxane[5',6'-*c*]xanthone, *trans*-(±)-2'-

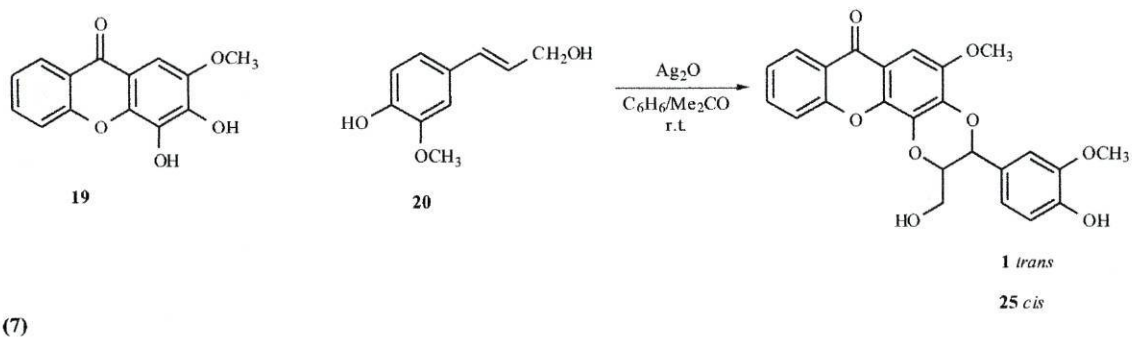


Fig. (7)

Table 4. Plants with Medicinal Applications Containing Xanthonolignoids

Species	Applications
Psorospermum febrifugum	Febrifuge, leprosy treatment, poison antidote and purgative [6]
	Antitumor and cytotoxic activities [17]
Vismia guaramiragnae	Purgative, tonic, febrifuge [16]
Calopyllum inophyllum	Treatment of rheumatism, skin diseases, dysentery and bleeding piles [61]
	Cicatrisant [62]
	Eye remedy [62]
Caraipa grandiflolia	Antihelminthic [63]
Cratoxylum cochinchinense	Treatment of fevers, coughs, diarrhea, itches, ulcers and abdominal complaints [64]
Kielmeyera coriacea	Antidepressant activity [65]
	Antifungal activity [52]
Harungana madagascariensis	Skin diseases, leprosy spots, and itch [66]
Hypericum mysorense	Antifungal activity [22]
Hypericum henryi	Treatment of infectious hepatitis [3]
Hypericum perfuratum	Antidepressive [67-69]
	Anti-inflammatory and antiviral properties [69]

hydroxymethyl-3'-(4"-hydroxy-3"-methoxyphenyl)-1',4'-dioxane[5',6'-*a*]xanthone (**28**), as well as *trans*-(±)-3'-hydroxymethyl-2'-(4"-hydroxy-3"-methoxyphenyl)-1',4'-dioxane[5',6'-*a*]xanthone (**29**), and *trans*-(±)-2'-hydroxymethyl-3'-(4"-hydroxy-3"-methoxyphenyl)-1',4'-dioxane[5',6'-*b*]xanthone (**30**). These compounds (**24**, **27-30**) are positional isomers and their structures can be grouped in the I/II, III, and V/VI frameworks carrying a significant tridimensional difference as "pistol", "linear" and "long chair" shapes, respectively (Fig. (**8**)) [27].

The investigation of the oxidative coupling reaction, in the presence of several oxidizing agents, revealed that the type of regio- and/or stereoisomers formed depends on the position of the hydroxyl groups in the xanthonic building block and that higher yields are obtained when potassium hexacyanoferrate (III) is used as oxidizing agent. Nevertheless, no differences are observed in the regio- and stereoselectivity of the reaction when different oxidizing agents are used. Based on the references [70,71], the conversion of the 1,4-benzodioxane *cis* isomer into the *trans* derivative was achieved for compound **27**, by the ring opening/closing treatment with potassium carbonate [27].

STRUCTURE ELUCIDATION

Xanthonolignoids are normally separated by chromatography on silica gel [1-3,12-14,16-19,20,21,23,24,52] or by HPLC [25,72] using different mobile phases, and can be identified by comparison with authentic samples by TLC [20,22,24] or by spectral analysis [20,22,24]. The structure of xanthonolignoids has been established by UV, IR, MS and mainly by NMR methods [1-3,7,14,16,17-21,23,24,52]. The increase of new and structurally diverse natural/synthetic xanthonolignoids along with the appearance of advanced NMR techniques has allowed the unambiguous structure determination in this class of compounds.

The *cis/trans* relationship of the substituents on the dioxane ring can be easily determined taking into

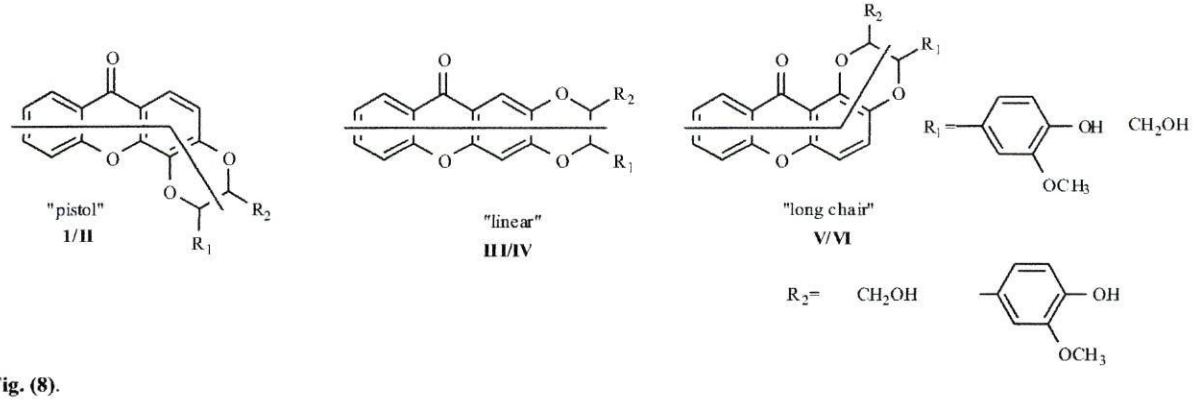


Fig. (8).

consideration the coupling constant $^3J_{H3\text{-}H2}$ in the ^1H NMR spectra of xanthonolignoids. Thus, a *trans* configuration for the two protons of the dioxane ring was established with coupling constant values $^3J_{H3\text{-}H2}$ =7.0-9.0 Hz for compounds **1-17**, **24**, **26**, **28-30**. On the other hand, coupling constant values of $^3J_{H3\text{-}H2}$ = 3.0 and 2.8 Hz for compounds **25** and **27** respectively, suggested a *cis* configuration. Furthermore, the proton resonances of 1,4-dioxane ring for *cis*-isomers show higher frequencies for H-3' and H-2' than in the *trans*-isomers, respectively. These stereochemistry assignments were confirmed by NOE experiments, upon irradiation of H-3' and/or H-2' [26,27].

One of the interesting aspects in the structure elucidation of xanthonolignoids was the determination of the substituents orientation on the 1,4-dioxane ring. The regioisomerism of the dioxane ring was determined for the first time in kielcorin (1) by the ring cleavage with NaOMe, leading to a phenolic group at C-3 of the xanthone moiety, in the *para* position to the carbonyl group. This cleavage produced a bathochromic shift in the UV spectrum by addition of alkali solution [14].

The major differences among the NMR spectra of xanthonolignoids are due to the proton and carbon resonances of the ring bearing the benzodioxane moiety. Thus, for example, by comparison of the pairs of *trans*-kielcorin B (**6**)/ *trans*-isokielcorin B (**26**) and *trans*-kielcorin D (**28**)/ *trans*-isokielcorin D (**29**) the downfield shifts (Table 5) are clearly caused by the strong deshielding mesomeric effects exerted by the carbonyl group, through the oxygen substituent on C-1' of both structures.

The orientation of the substituents on the 1,4-dioxane rings was also unequivocally established by long-range C-H connectivities using the one-dimensional selective INEPT technique [23,26,27].

BIOLOGICAL ACTIVITIES

Data for the biological activities of xanthonolignoids are sparse [17,28,31]. The study of the cytotoxic activity for the xanthonolignoids **1**, **5** and **12**, isolated from *Psorospermum febrifugum*, on A 549 (human lung carcinoma), MCF-7 (breast carcinoma), and HT-29 (human colon adenocarcinoma) showed that they had very low activity [17]. Later, the studies on the inhibitory activity against DNA topoisomerase I and II, by 6-hydroxycadensin F (**11**), showed no significant potency [49]. The hepatoprotective effect of the xanthonolignoids *trans*-kielcorin (**1**), *trans*-isokielcorin B (**25**), and their xanthonic building blocks against *tert* - butylhydroperoxide - induced toxicity in isolated rat hepatocytes was very promising, when compared with the result obtained for sylibin [28].

Consequently, we have tested eight isomeric xanthonolignoids **1**, **6**, **22**, **24-28** and four xanthonic building blocks, 1,2-, 2,3- and 3,4-dihydroxyxanthones, and 2,3-dihydroxy-4-methoxyxanthone for their *in vitro* cytotoxic activity against three human tumor cell lines: MCF-7 (breast), TK-10 (renal), and UACC-62 (melanoma) using the sulforhodamine B method for the determination of cellular growth and viability. These compounds were also evaluated for their effect on the mitogenic response of human T-lymphocytes to PHA using a colorimetric MTT assay [27]. Though these activities varied considerably with the structures of the compounds tested, we have found that, in general, best results were associated with "linear" molecules and the *trans* isomers. Using the natural compounds **1** and **6** as models, it was found that the absence of the methoxy group on the xanthonic nucleus caused an increase of growth inhibitory activity.

CONCLUSION

This review has provided the current knowledge on chemistry and biological activities of xanthonolignoids. Natural compounds are very interesting templates for molecular modifications in order to improve physicochemical and biological properties.

The knowledge gained and the results of some biological assays can contribute to future rational design of new pharmacological useful compounds, based on kielcorin framework.

ACKNOWLEDGEMENTS

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Table 5. Values of δ for H-2'/H-3' and C-2'/C-3' for Kielcorins and Isokielcorins

	δ			
	6	**26**	**28**	**29**
H-2'	4.40 (m)	4.53 (m)	4.22 (m)	4.33 (m)
H-3'	5.24 (d, J=8.3)	5.13 (d, J=8.0)	5.05 (d, J=7.6)	5.00 (d, J=7.8)
C-2'	77.8	77.8	77.2	78.0
C-3'	76.9	75.6	76.1	75.3

ABBREVIATIONS

PHA = Phytohemagglutinin

MTT = 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

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ANEXO II

Isomeric Kielcorins and Dihydroxyxanthones: Synthesis, Structure Elucidation, and Inhibitory Activities of Growth of Human Cancer Cell Lines and on the Proliferation of Human Lymphocytes *In Vitro*

by **Emília P. Sousa**[a][b], **Artur M. S. Silva**[c], **Madalena M. M. Pinto***[a], **Madalena M. Pedro**[a], **Fátima A. M. Cerqueira**[a], and **Maria S. J. Nascimento**[a]

[a]) Centro de Estudos de Química Orgânica, Fitoquímica e Farmacologia da Universidade do Porto, Faculdade de Farmácia, Rua Aníbal Cunha, 164, 4050-047, Porto, Portugal (fax: +351-222057358; e-mail: madalena@ff.up.pt)

[b]) Instituto Superior Ciências da Saúde-Norte, Rua Central da Gandra, 1317, 4580 Gandra, Portugal

[c]) Departamento de Química, Universidade de Aveiro, 3810-193 Aveiro, Portugal

The synthesis, structure elucidation, and biological activities of five isomeric xanthonolignoids, (±)-*trans*-kielcorin C, (±)-*cis*-kielcorin C, (±)-*trans*-kielcorin D, (±)-*trans*-isokielcorin D, and (±)-*trans*-kielcorin E, are reported. The synthetic approach is based on the oxidative coupling of coniferyl alcohol with an appropriate xanthone. The influence of different oxidizing agents was studied, and the best results were obtained with potassium hexacyanoferrate(III). The structure elucidation was achieved by 2D-NMR techniques such as COSY, HETCOR, HSQC, and HMBC. Long-range C,H connectivities were used to establish the orientation of the substituents on the 1,4-dioxine rings, while NOE experiments were used to determine the configurations of these rings. These xanthonolignoids, as well as (±)-*trans*-kielcorin, (±)-*trans*-kielcorin B, (±)-*trans*-isokielcorin B, and the xanthonic building blocks 3,4-, 1,2-, and 2,3-dihydroxy-9*H*-xanthen-9-ones, and 2,3-dihydroxy-4-methoxy-9*H*-xanthen-9-one, were evaluated for their *in vitro* effect on the growth of three human cancer cell lines, MCF-7 (breast), TK-10 (renal), and UACC-62 (melanoma), and on the proliferation of human lymphocytes.

1. Introduction. – Natural kielcorins are xanthonolignoids isolated from several plants of Clusiaceae [1]. The biomimetic synthesis of kielcorin (**1**) [2] and kielcorin B (**2**) [3], as well as isokielcorin B (3) [3] (*Fig. 1*), by oxidative coupling of 3,4-dihydroxy-2-methoxy-9*H*-xanthen-9-one (**4**) and 2,3-dihydroxy-4-methoxy-9*H*-xanthen-9-one (**5**), respectively, with coniferyl alcohol (=4-(3-hydroxyprop-1-enyl)-2-methoxyphenol; **6**) was previously achieved (*Fig. 2*). These kielcorins, as well as their xanthonic building blocks, were assayed for their biological activities and exhibited interesting hepatoprotective activity against (*tert*-butyl)hydroperoxide-induced toxicity in isolated rat hepatocytes [4].

To obtain related bioactive compounds with a kielcorin framework, five constitutional isomers were designed. In this paper, the synthesis of *trans*- and *cis*-isomers of (±)-2,3-dihydro-3-(4-hydroxy-3-methoxyphenyl)-2-(hydroxymethyl)-7*H*-1,4-dioxino-[2,3-*c*]xanthen-7-one, **7** and **8**, respectively, and the (±)-*trans*-2,3-dihydro-3-(4-hydroxy-3-methoxyphenyl)-2-(hydroxymethyl)-12*H*-1,4-dioxino[2,3-*a*]xanthen-12-one (**9**), as well as (±)-*trans*-2,3-dihydro-2-(4-hydroxy-3-methoxyphenyl)-3-(hydroxymethyl)-12*H*-1,4-dioxino[2,3-*a*]xanthen-12-one (**10**), and (±)-*trans*-2,3-dihydro-3-(4-hydroxy-3-methoxyphenyl)-2-(hydroxymethyl)-11*H*-1,4-dioxino[2,3-*b*]xanthen-11-one (**11**) are reported (*Fig. 1*). The synthetic approach, based on a biomimetic pathway as a model,

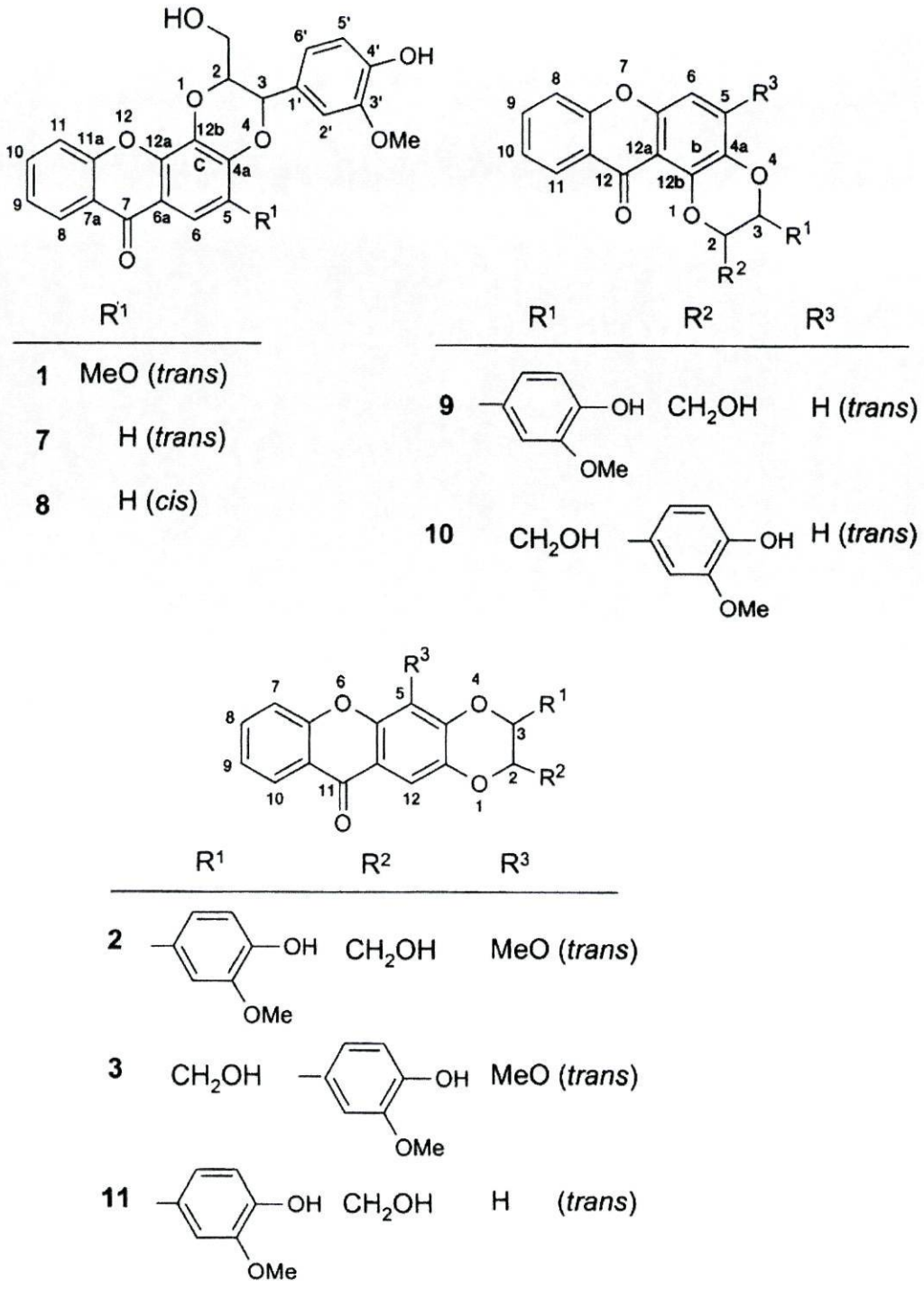
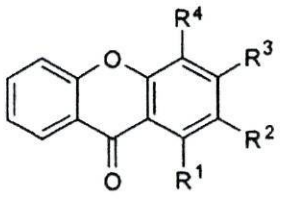
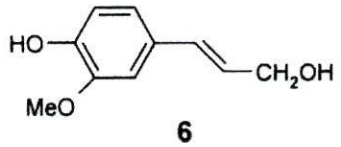


Fig. 1. *Structures of kielcorins* **1**–**3** *and* **7**–**11**

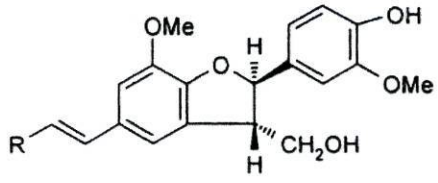
was by oxidative coupling of coniferyl alcohol (**6**) with an appropriate xanthone, 3,4-dihydroxy- (**12**), 1,2-dihydroxy- (**13**), and 2,3-dihydroxy-9*H*-xanthen-9-one (**14**), for the C, D, and E series of kielcorins, respectively (*Scheme 1*). Different oxidizing agents were used (*e.g.*, Ag_2O, Ag_2CO_3, and $K_3[Fe(CN)_6]$) to investigate the oxidative coupling reaction [5]. The total synthesis of demethoxykielcorin **7** has already been accomplished in low yield by a multi-step pathway [6]. Herein, the *cis*-isomer **8** was efficiently converted to the *trans*-isomer **7** [7].





6

	R^1	R^2	R^3	R^4
4	H	MeO	OH	OH
5	H	OH	OH	MeO
12	H	H	OH	OH
13	OH	OH	H	H
14	H	OH	OH	H



21 R=CHO

22 R= CH_2OH

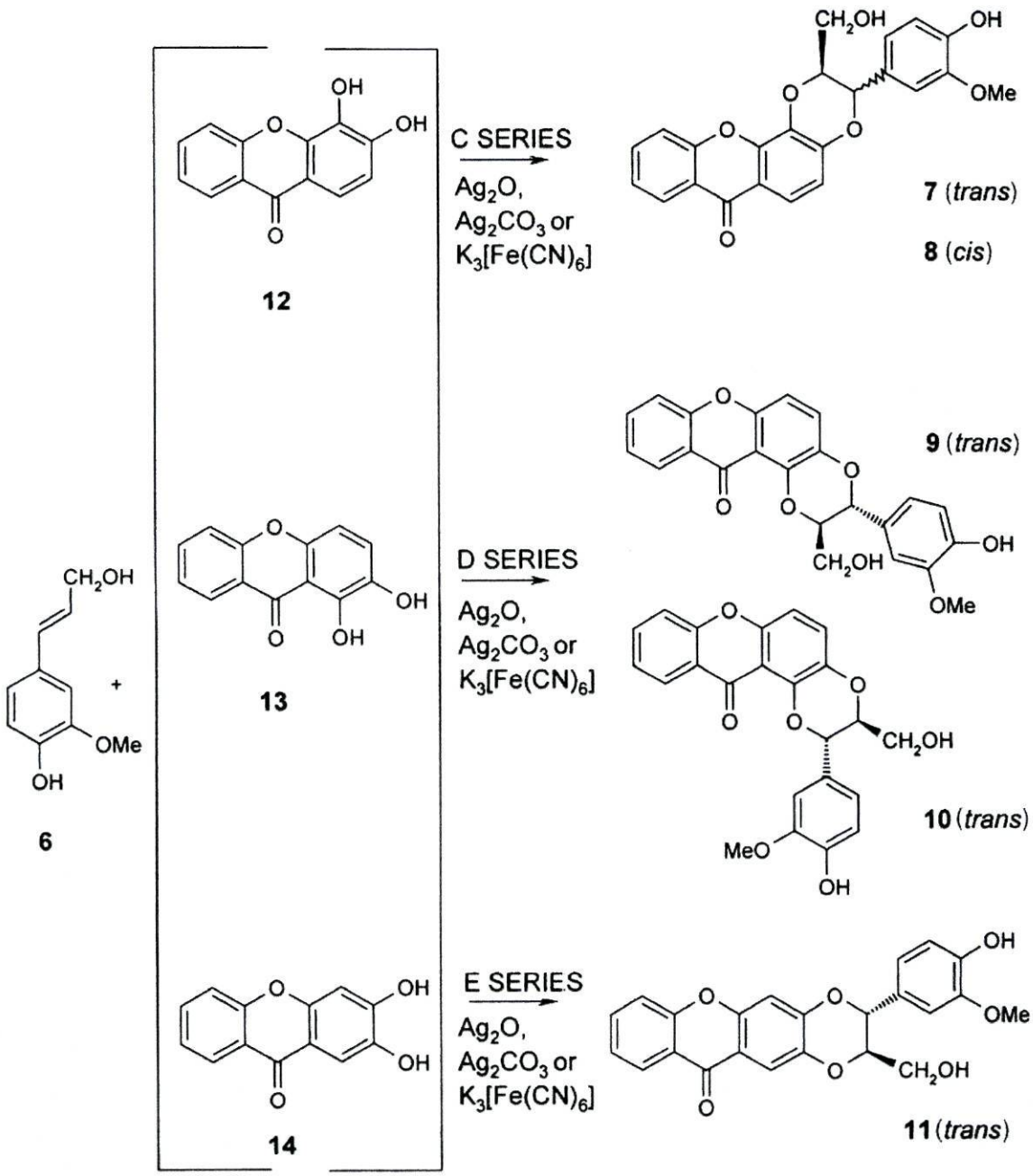
Fig. 2. *Structures of xanthones* **4**, **5** *and* **12**–**14**, *coniferyl alcohol* (**6**), *and the dimeric products* **21** *and* **22**

Few data are available for biological activities of kielcorins [4][8]. In this work, results of the effect of kielcorin derivatives **1**–**3** and **7**–**11**, and xanthones **5** and **12**–**14** on the growth of three human cancer cell lines MCF-7 (breast), TK-10 (renal), and UACC-62 (melanoma), as well as on the proliferation of human lymphocytes are described.

2. Results and Discussion. – 2.1. *Synthesis of Kielcorin Derivatives with Several Oxidizing Agents.* In a series of studies on the synthesis of bioactive lignans, flavonolignoids, and coumarinolignoids, several authors have investigated oxidative coupling reactions [5][9]. For simpler compounds, it was shown that the substituents on the catechol ring had a striking influence on the regioselectivity of the reaction; it was highly regioselective in cases where the catechol skeleton possessed electron-donating substituents and much less regioselective with catechols bearing electron-withdrawing substituents [9]. A free-radical coupling mechanism, with the first step being the intermolecular *O*-*β* coupling of two phenoxyl radicals, has been suggested [10]. Further studies on the synthesis of model compounds bearing a 3-aryl- or 2-aryl-1,4-benzodioxin skeleton achieved high regioselectivity by oxidative coupling in the presence of an Ag salt (oxide, carbonate) or $K_3[Fe(CN)_6]$, respectively [5a]. Based on these studies, an investigation was initiated to explore the regio- and stereoselectivity of the reaction with xanthones as one of the building blocks of benzodioxine rings that bear a C=O function as an electrophilic group and a heteroatom with electron-donating properties. It appeared reasonable to investigate the oxidative coupling in the presence of the same oxidizing agents (Ag_2O, Ag_2CO_3, and $K_3[Fe(CN)_6]$).

The synthesis of 1,2- and 2,3-dihydroxy-9*H*-xanthen-9-one, **13** and **14**, respectively (*Scheme 2*), involved as starting materials methyl 2-bromobenzoate (**15**) and 3,4-dimethoxyphenol (**16**); from the resulting methyl 2-(3,4-dimethoxyphenoxy)benzoate (**17**) [11], after hydrolysis and subsequent cyclization of 2-(3,4-dimethoxyphenoxy)-benzoic acid (**18**) with lithium diisopropylamide (LDA) [12] or with AcCl [13],

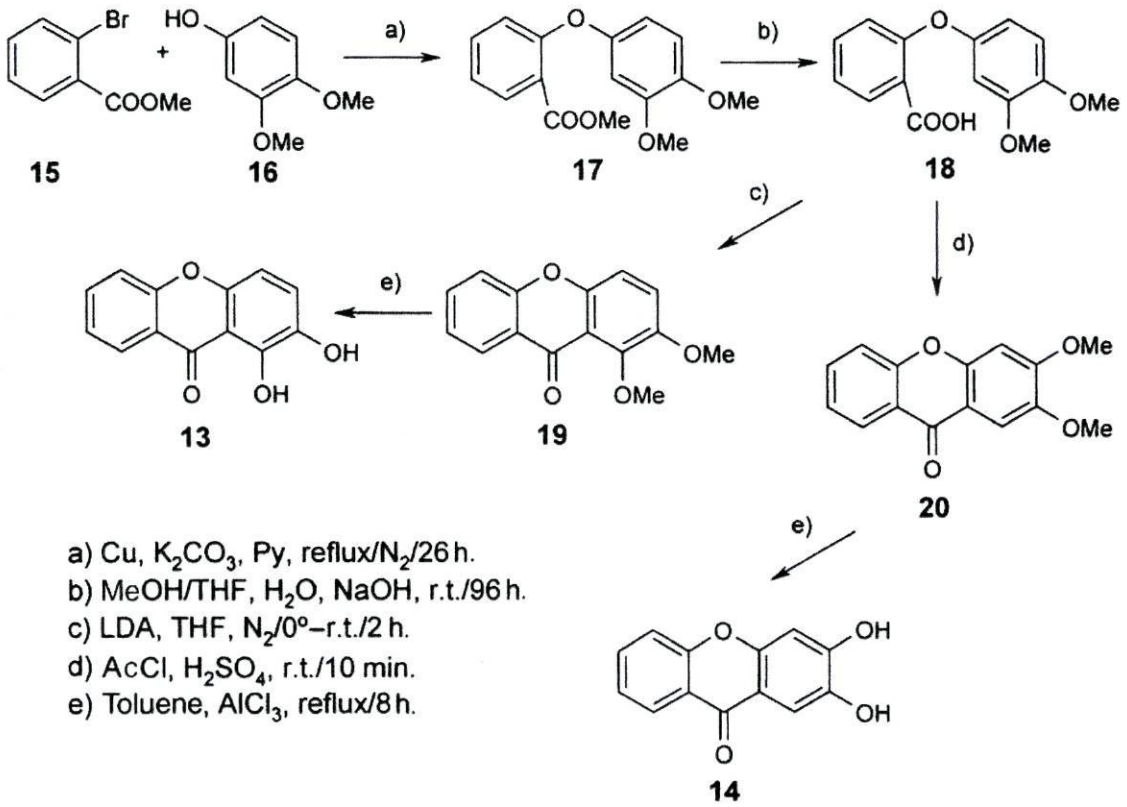
Scheme 1



compounds **19** and **20**, respectively, were obtained and demethylated to afford **13** and **14**, respectively. 3,4-Dihydroxy-9*H*-xanthen-9-one (**12**) was synthesized by a method described in [14]. For compounds **12**–**14**, and **20** are provided, for the first time, spectral data from INEPT, HETCOR, COSY, or HMBC experiments to clarify the structural elucidation of these compounds.

The oxidative coupling of the appropriate dihydroxyxanthones **12**–**14** and an equimolar amount of coniferyl alcohol (**6**) were carried out at room temperature, with 1.5 mol equiv. of Ag_2O or Ag_2CO_3, in toluene/Me_2CO, for 2–3 days. In the case of the other oxidizing agent, equimolar amounts of **12**–**14** and **6** were treated with 20 mol-equiv. of $K_3[Fe(CN)_6]$, at room temperature, in a mixture of Me_2CO/H_2O and an aqueous 4% AcONa solution (*Scheme 1*).

Scheme 2



The xanthonolignoid distribution in the crude products of the oxidative coupling reactions under the conditions indicated above is summarized in *Table 1*. The isomeric nature of the kielcorin derivatives obtained did not differ with the oxidizing agents: for the C series, (±)-*trans*-kielcorin C (**7**) and (±)-*cis*-kielcorin C (**8**) were obtained for all the oxidizing agents; the D series gave two isomeric xanthonolignoids, (±)-*trans*-kielcorin D (**9**) and (±)-*trans*-isokielcorin D (**10**); and the E series afforded (±)-*trans*-kielcorin E (**11**). The regioselectivity achieved with Ag salts or with $K_3[Fe(CN)_6]$ for simpler compounds was not verified for compounds **7**–**11**. In the C series, none of the oxidizing agents used were stereoselective, although, when Ag_2O and Ag_2CO_3 was used, a lower amount of the *cis*-isomer **8** was obtained. Two dimeric compounds, phenylcoumaran **21** and dehydrodiconiferyl alcohol **22**, were also found as by-products (*Fig. 2*) in all reactions [3]. By carrying out the oxidative coupling with $K_3[Fe(CN)_6]$, the quantities of **21** and **22** were markedly reduced, and a higher yield of the kielcorins was obtained.

The most efficient method involved the use of $K_3[Fe(CN)_6]$ as oxidizing agent in the oxidative coupling reaction (*Table 1*). Therefore, in the C series, the mixture of isomer **7** (*trans*) and **8** (*cis*) thus obtained was treated with anhydrous K_2CO_3 [7] to yield isomer **7**, exclusively (*Scheme 3*).

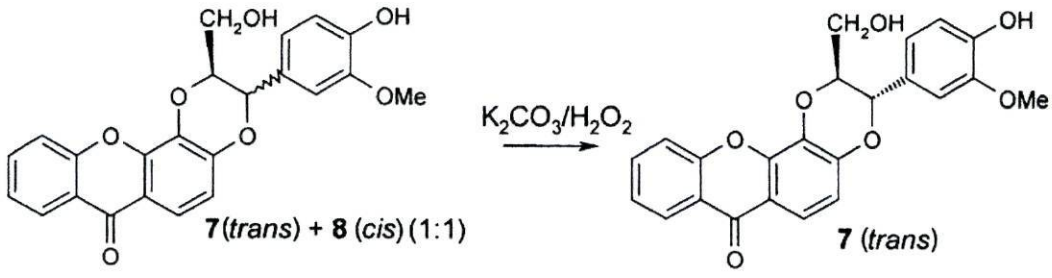
The position of the OH substituents had a striking effect on the nature of the kielcorin isomers obtained: when 3,4-dihydroxy-9*H*-xanthen-9-one (**12**) was used as a building block, a mixture of isomers **7** (*trans*) and **8** (*cis*) was formed; with 1,2-dihydroxy-9*H*-xanthen-9-one (**13**), the *trans*-isomers **9** and **10** were obtained, whereas 2,3-dihydroxy-9*H*-xanthen-9-one (**14**) gave only the *trans*-isomer **11**. The electron-

Table 1. *Distribution of Xanthonolignoids* **7**–**11** *in the Crude Reaction Products*

Compound	Yields [%]		
	Ag_2O	Ag_2CO_3	$K_3[Fe(CN)_6]$
7[a])	20	17	29
8[a])	3.5	3.0	11
9[b])	22	20	27
10[b])	0.7	0.4	0.8
11[c])	3.0	2.0	4.0

[a])[b])[c]) HPLC conditions: C_{18}-*Nucleosil* column, 25 , 1 ml/min. [a]) MeOH/1% AcOH in H_2O 6:4, 239 nm. [b]) MeCN/1% AcOH in H_2O 5:5, 239 nm. [c]) MeOH/1% AcOH in H_2O 7:3, 241 nm.

Scheme 3

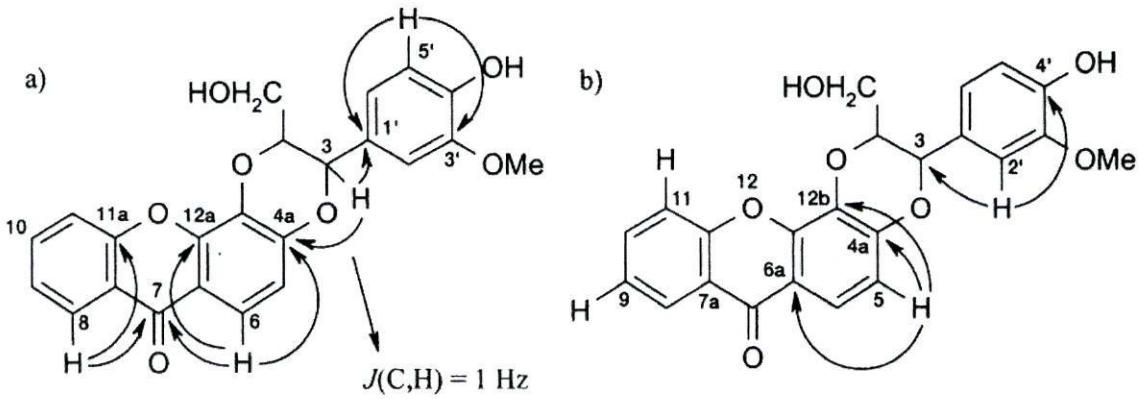


withdrawing and electron-donating effects of the C=O group, and the heteroatom of these xanthones **12**–**14** could influence the electronic character of the radical formed on positions 3,4, 1,2, and 2,3 of the xanthone in different ways. The involvement of the olefinic C=C bound in the formation of an Ag complex can also be an explanation for this kind of oxidative coupling reaction and deserves further investigation.

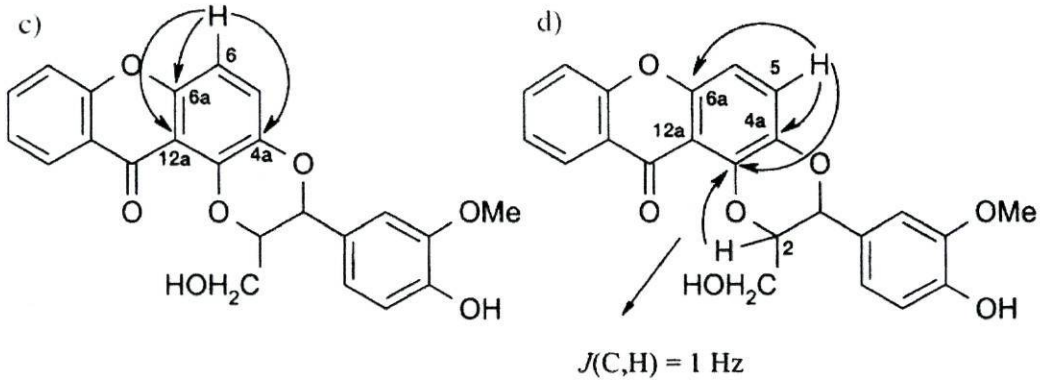
2.2. *Structure Elucidation of Kielcorins.* The analysis of the ^{1}H- and COSY-NMR spectra of xanthonolignoids **7**–**11** allowed the assignment of the *ABCD* spin system corresponding to the resonances of H–C(8), H–C(9), H–C(10), and H–C(11) (in **7**–**10**) and H–C(7), H–C(8), H–C(9), and H–C(10) (in **11**). H–C(8) (H–C(11) in **9** and **10** and H–C(10) in **11**) (δ 8.04–8.20 ppm) is the more deshielded of these protons, due to the mesomeric and anisotropic deshielding effect of the C=O group, followed by H–C(10) in **7** and **8**, H–C(9) in **9** and **10**, and H–C(8) in **11** (δ 7.80–7.87 ppm), which only feels the mesomeric effect. The assignment of the corresponding ^{13}C resonances were made from the correlations found in the HETCOR or HSQC spectra, whereas those of the quaternary C-atoms of the unsubstituted ring were based on the connectivities found in the HMBC and on the one-dimensional selective INEPT experiments [15] of these compounds (H–C(8) → C(11a), C(7), C(10); H–C(11) and H–C(9) → C(7a)) (**a** and **b**; *Fig. 3*).

Due to the presence of electron-donating substituents on the 1,3,4-trisubstituted phenyl rings, originated from the coniferyl alcohol, their ^{1}H resonances were the more shielded ones (H–C(2′): 7.04–7.10 ppm; H–C(5′): 6.80–6.83 ppm; H–C(6′): 6.89–6.90 ppm). The corresponding protonated ^{13}C resonances were assigned based on the correlations found in the HETCOR or HSQC spectra, whereas those of the quaternary C-atoms were based on the connectivities found in their HMBC spectra (H–C(5′) and H–C(3) → C(1′); H–C(5′) → C(3′); H–C(2′) → C(4′) and C(3) (**a** and **b**; *Fig. 3*).

(±)-*trans*-kielcorin C (**7**)



(±)-*trans*-kielcorin D (**9**)



(±)-*trans*-kielcorin E (**11**)

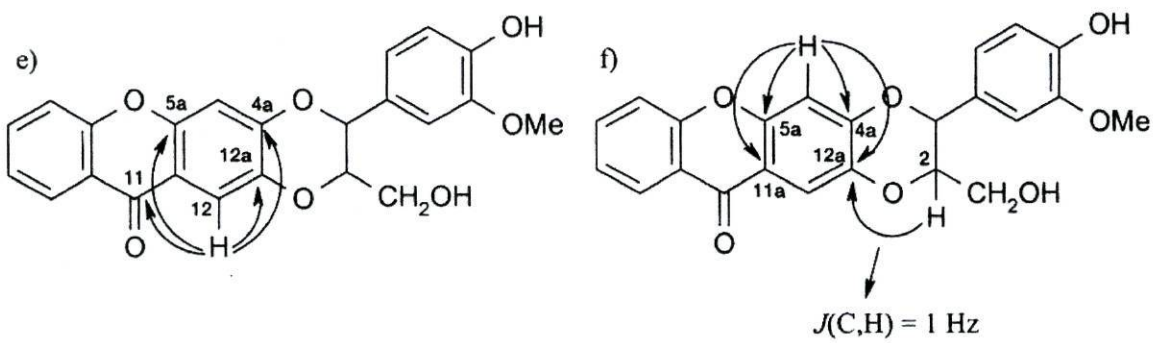


Fig. 3. *Main connectivities found in the HMBC and in the one-dimensional selective INEPT experiments of xanthonolignoids* **7**, **9**, *and* **11**

The major differences among the NMR spectra of the xanthonolignoids synthesized are due to the ^1H and ^{13}C resonances of the ring bearing the 'benzodioxane' moiety. In the case of (±)-*trans*-kielcorin C (**7**), H–C(5) and H–C(6) appear as two *doublets*, and the connectivities found in the HMBC allowed the assignment of the quaternary C-atoms C(4a), C(12a), C(12b), and C(6a) as depicted in *Fig. 3* (*a* and *b*). However, the

unequivocal identification of the structure of compound **7** as (±)-*trans*-kielcorin C was accomplished from the one-dimensional selective INEPT [15]. Upon H–C(3) irradiation, with a long-range C,H coupling constant optimized for 1 Hz [15b], enhancements on the C(4a) resonance was observed, allowing the establishment of the connection of the 1,4-dioxane moiety to the xanthone ring. The assignments of all of the ^{1}H and ^{13}C resonances of the substituted ring of the compounds **8**–**11**, and the connection of their 1,4-dioxane moiety were deduced by a similar procedure (*c*–*f*, *Fig. 3*).

In the case of (±)-*trans*-isokielcorin D (**10**), the one-dimensional selective INEPT did not allow the establishment of any connectivity between H–C(2) or H–C(3), and the C-atoms of the xanthone ring. However, a comparison of the ^{1}H and ^{13}C resonances on 1,4-dioxane ring with those of (±)-*trans*-kielcorin D (**9**) shows the downfield shifts of C(2) of **10** relative to C(3) of **9**. Comparable downfield shifts of C(2) of **9** relative to C(3) of **10** are also observed (*Fig. 4*). In both cases, the downfield shifts are clearly caused by the strong deshielded mesomeric effect exerted by the C=O group, through the O-substituent at C(12b) of both structures [3].

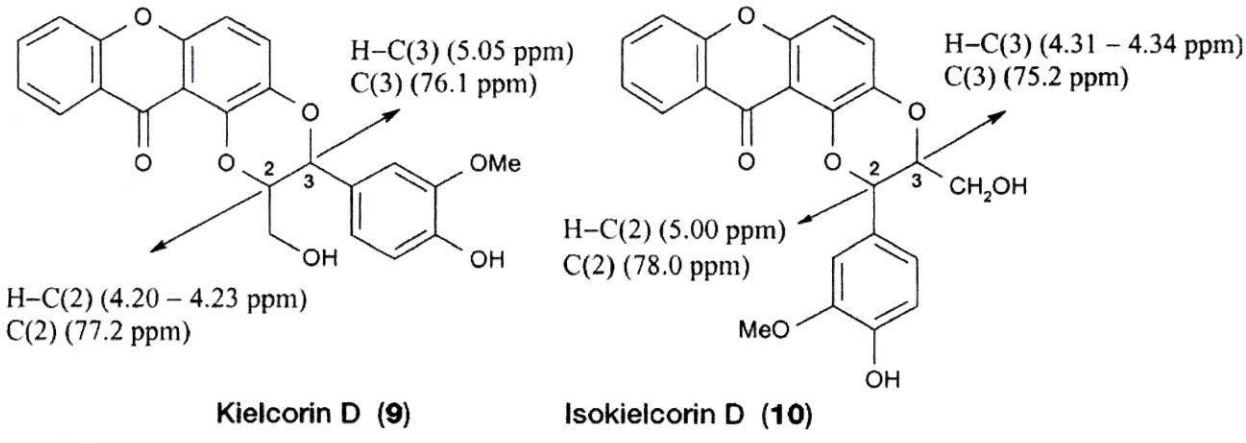


Fig. 4. *Comparison of the ^{1}H and ^{13}C resonances of the 1,4-dioxane of (±)*-trans-*kielcorin D* (**9**) and (±)-trans-*isokielcorin D* (**10**)

Taking into consideration the coupling constant values $^3J(2,3) = 7.6–8.1$ Hz in the ^{1}H-NMR spectra of xanthonolignoids **7**, **9**–**11**, and our previous report [3] allowed the establishment of a *trans*-configuration for these two H-atoms. The absence of a NOE effect between these two H-atoms supported this configurational assignment.

In the course of the synthesis of *trans*-kielcorin C (**7**), another compound was isolated with similar structural features. The main difference is the coupling constant value of $^3J(2,3) = 2.8$ Hz, which suggests a *cis*-configuration for these two H-atoms. This configuration was confirmed by the observed NOE enhancement (6%) in the H–C(3) resonance on irradiation of H–C(2), supporting their close proximity. This compound is named *cis*-isokielcorin C (**8**).

2.3. *Biological Activities. Table 2* summarizes the *in vitro* effects of (±)-*trans*-kielcorin (**1**), (±)-*trans*-kielcorin B (**2**), (±)-*trans*-isokielcorin B (**3**), (±)-*trans*-kielcorin C (**7**), (±)-*cis*-kielcorin C (**8**), (±)-*trans*-kielcorin D (**9**), (±)-*trans*-isokielcorin D (**10**), (±)-*trans*-kielcorin E (**11**) and of the four xanthonic building blocks 2,3-dihydroxy-4-methoxy-9*H*-xanthen-9-one (**5**), 3,4-, 1,2-, and 2,3-dihydroxy-

Table 2. *Effects of Xanthone derivatives* **1**–**3**, **5**, *and* **7**–**14** *on the Growth of Human Cancer Cell Lines and Proliferation of Human Lymphocytes*

Compound	GI_{50} [μM][a])[b])			IC_{50} [μM][a])[c])
	MCF-7 (Breast)	TK-10 (Renal)	UACC-62 (Melanoma)	Human lymphocytes
1	53.5 ± 8.0	72.1 ± 5.6	36.6 ± 2.4	> 100
5	37.2[d])	76.6 ± 7.0	19.8 ± 1.6	17.4 ± 2.7
2	62.5 ± 13.3	88.5 ± 8.6	80.8 ± 11.6	> 100
3	16.2 ± 3.4	30.9 ± 5.0	25.4 ± 1.3	41.9 ± 1.6
12	40.5 ± 1.5	59.2[d])	21.6 ± 2.6	12.2 ± 1.3
7	18.3 ± 0.7	25.9 ± 2.7	26.2 ± 1.3	36.2 ± 7.4
8	32.0 ± 2.4	45.2 ± 7.8	33.3 ± 2.1	48.6 ± 6.1
13	38.4 ± 2.7	65.8 ± 5.1	14.0 ± 0.3	73.3 ± 2.2
9	> 100	97.5 ± 10.5	> 100	> 100
10	82.8 ± 4.3	> 100	> 100	> 100
14	40.6 ± 1.3	61.4 ± 4.3	31.7 ± 3.7	31.2 ± 1.5
11	21.2 ± 8.2	23.1 ± 0.5	21.9 ± 3.9	> 100

[a]) Results are expressed as GI_{50} (concentrations of compounds that cause 50% inhibition of cancer cell growth) or IC_{50} (concentrations that cause 50% inhibition of lymphocytes proliferation), and show means ± SEM of 3–6 independent observations performed in duplicate. [b]) Doxorubicin was used as positive control in cancer cell lines growth (GI_{50} (MCF-7) = 42.8 ± 8.2 nM; GI_{50} (TK-10) = 548.0 ± 60.0 nM; GI_{50} (UACC-62) = 94.0 ± 9.4 nM). [c]) Cyclosporin A was used as positive control in lymphocyte proliferation (IC_{50} = 0.34 ± 0.04 μM). [d]) Data based on two independently run duplicate experiments.

9*H*-xanthen-9-ones **12**–**14** on the growth of the human cancer cell lines MCF-7, TK-10, UACC-62, and on the proliferation of human lymphocytes.

2.3.1. *Effect on the Growth of Human Cancer Cell Lines.* An inhibition of the growth of the human cancer cell lines was observed with almost all kielcorins and oxygenated xanthones studied. The growth inhibitory effect was, in general, moderate but was shown to be dose-dependent and due to growth arrest and not to cell death, as inferred from the sulforhodamine B (SRB) assay.

Considering the selectivity, the results in *Table 2* showed that 2,3-dihydroxy-4-methoxy-9*H*-xanthen-9-one (**5**), and 3,4- and 1,2-dihydroxy-9*H*-xanthen-9-ones (**12** and **13**, resp.) are significantly ($P < 0.05$) more active against UACC-62 than to MCF-7 and TK-10 cell lines.

The comparison between the effects of 3,4- and 2,3-dihydroxy-9*H*-xanthen-9-ones (**12** and **14**, resp.) with those of the (±)-*trans*-kielcorin derivatives **7** and **11**, respectively, revealed a significant ($P < 0.05$) increase of activity in MCF-7 and TK-10 cell lines. No significant difference was observed for UACC-62 ($P > 0.05$). On the contrary, compounds **2** and **9** were associated with a decrease and loss of growth-inhibitory activity, respectively, in all of the cancer cell lines when compared with their xanthonic building blocks **5** and **13**.

The isomerism of the tested kielcorins **7**–**11** seemed to influence the growth-inhibitory activity of the human cancer cell lines: the kielcorins of the C (**7** and **8**) and E (**11**) series were significantly more active ($P < 0.005$) than their D isomers (**9** and **10**). In the *cis*/*trans* pair, it is interesting to note that (±)-*trans*-kielcorin C (**7**) was shown to be a more potent inhibitor than its *cis*-isomer **8** ($P < 0.05$). Also, (±)-*trans*-isokielcorin

B (**3**) was associated with a significantly ($P < 0.05$) stronger effect compared with its constitutional isomer (±)-*trans*-kielcorin B (**2**).

It is also interesting to note that the absence of the MeO substituent in the xanthonic framework was associated with a significant ($P < 0.05$) increase of growth inhibitory activity as revealed by kielcorins **7** and **11**, relative to those of **1** and **2**, respectively.

2.3.2. *Effect of Kielcorins on Human Lymphocytes Proliferation.* Inhibition of human lymphocyte proliferation induced by phytohemagglutinin (PHA) was detected with all of the oxygenated xanthones **5** and **12**–**14**, and with (±)-*trans*-isokielcorin B (**3**) and the kielcorins in C series, **7** and **8**. The antiproliferative activity detected was dose-dependent and could not be attributed to a toxic effect on lymphocytes as demonstrated by the lymphocytoxicity assay (viability of exposed lymphocytes > 70%). (±)-*trans*-Kielcorin (**1**) and compounds in D and E series, **9**–**11**, showed no inhibitory effect even when tested at 100 μM.

Comparing the antiproliferative activity of the dihydroxy-9*H*-xanthen-9-ones, it was concluded that the position of the *ortho*-dihydroxy groups influence the suppresser effect of these compounds (*Table 1*). Positions 3,4- were associated with a significantly ($P < 0.01$) stronger antiproliferative effect when compared with the 1,2- and 2,3-positions. Kielcorins **3**, **7**, and **8** exhibited a significantly ($P < 0.05$) less-potent suppresser effect than that of their xanthonic building blocks **5** and **12**.

3. Conclusions. – In summary, the synthesis of five new structures based on models of natural kielcorins is reported. For this kind of compound, the most efficient method in the oxidative coupling reaction involves the use of $K_3[Fe(CN)_6]$ as oxidizing-agent. Long-range C,H connectivities and NOE experiments permit unambiguous establishment of the structures of the different isomers. The compounds of the C and E series are the most active for the inhibition of the growth activity. For the inhibition of human lymphocyte proliferation, generally, the kielcorins are less potent than their xanthonic building blocks. The differences in the isomerism and in the nature of the substituents in the xanthonolignoid framework will allow us to evaluate the structure features, and further modifications are underway to improve the activity of these compounds.

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Experimental Part

1. *General.* Column chromatography (CC): *Merck* silica gel *60* (0.50–0.20 mm). Flash chromatography (FC): *Merck* silica gel *60* (0.040–0.063 mm). Anal. and prep. TLC: *Merck* silica gel *60* (GF_{254}). The reactions were monitored by TLC ($CHCl_3$/MeOH) and interrupted, when one of the reagents was no longer detectable by TLC. Ag_2CO_3, AcONa, and $K_3[Fe(CN)_6]$ were supplied by *Merck* (D-Darmstadt). Coniferyl alcohol and Ag_2O were obtained from *Sigma* (D-Steunheim). Lithium diisopropylamide (LDA) soln. was purchased from *Fluka* (D-Neu-Ulm). Structure elucidations of phenylcoumaran (**21**) and dehydrodiconiferyl alcohol (**22**; *Fig. 2*) were accomplished by comparison with authentic samples. Reversed-phase (RP) HPLC: *Jasco 880-PU*, equipped with a *Jasco 875-UV* detector, and a *CSW 1.7* integrator; *Nucleosil-C_{18}* column (250 × 4.6 mm, 5 μm; *Macherey-*

Nagel, D-Düren) equipped with a guard pre-column (*Macherey-Nagel*); linear gradient for compounds **7** and **8** with MeOH/1% AcOH in H_2O 6:4, for compounds **9** and **10** with MeCN/1% AcOH in H_2O 5:5, and for compound **11** with MeOH/1% AcOH in H_2O 7:3; at 25 °; flow rate of 1 ml/min; sample injection volume 20 µl; detection at 239 nm for kielcorins **7**–**10** and 241 nm for kielcorin **11**; t_R in min. M.p. *Kofler* microscope; uncorrected. The optical rotation was zero for compounds **7**–**11** and was obtained on a *Polartronic Universal* polarimeter. IR Spectra: *Perkin-Elmer 257* spectrophotometer; KBr microplates; in cm^{-1}. UV Spectra: *Varian CARY 1E* spectrophotometer; λ_{max} in nm. ^{1}H- and ^{13}C-NMR Spectra: *Bruker AC-200, DRX-300*, or *DRX-500* spectrometer; at r.t., $CDCl_3$ or (D_6)DMSO soln.; δ in ppm relative to Me_4Si as an internal reference; coupling constants J in Hz. ^{1}H-NMR assignments were made by means of 2D COSY experiments, while ^{13}C-NMR assignments were made by 1D selective INEPT and 2D HETCOR, HSQC, and HMBC experiments (long-range C,H coupling constants were optimized to 7 and 1 Hz). NOE Experiments were conducted by means of NOE difference technique, with an irradiation time of 2 s and a relaxation delay of 4 s. EI-MS Spectra: *Hitachi Perkin-Elmer RMU-6M* spectrometer, in *m/z*. HR-MS Spectra: *VG AutoSpec-Q* spectromer; recorded as EI (electronic impact) mode.

Fetal bovine serum and *RPMI-1640* medium were obtained from *Gibco BRL*. Cyclosporin A, DMF, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), DMSO, doxorubicin hydrochloride, gentamicin, L-glutamine, *Histopaque-1077*, phytohemagglutinin (PHA), sodium lauryl sulfate (SDS), and sulforhodamine B (SRB) were purchased from *Sigma*. *National Cancer Institute* (Bethesda, MD, USA) kindly provided the cancer cell lines. Stock solns. of compounds were prepared in DMSO and stored at −20 ° providing uniform samples for retesting. These frozen concentrates were then diluted to the desired final concentrations with the appropriate diluents immediately prior the biological assays. Final concentrations of DMSO showed no interference with the biological activities tested. Unpaired Student's *t*-tests were used. Differences with *P* values below 0.01 or 0.05 were considered statistically significant. Whenever $GI_{50} > 100$ µM, values of 100 µM were considered to evaluate the statistical significance of differences.

2. *Synthesis of Kielcorins* **7**–**11**. *General Procedure for the Oxidative Coupling Reaction of Dihydroxy-9H-xanthen-9-ones* **12**–**14** *and Coniferyl Alcohol* (**6**). *Method A: with* a) Ag_2O *and* b) Ag_2CO_3. The appropriate dihydroxyxanthones **12**–**14** (615 mg, 2.7 mmol) and **6** (500 mg, 2.7 mmol) were taken up in toluene/Me_2CO 1:1 (50 ml) and stirred in the presence of 3.1 mmol of the oxidizing agent (*a*) 815 mg of Ag_2O or *b*) 860 mg of Ag_2CO_3), at r.t., in the dark for 2–3 days. The suspension was filtered, the filtrate evaporated, and the crude product was analyzed by HPLC. The crude reaction products were purified by CC with a mixture of hexane/$CHCl_3$/MeOH (several proportions). The isolation of the components was then carried out by prep. TLC ($CHCl_3$/MeOH 95:5). HPLC: t_R (% yield Ag_2O, Ag_2CO_3): 17.2 (20, 17) **7**, 20.2 (3.5, 3.0) **8**, 4.9 (22, 20) **9**, 8.4 (0.7, 0.4) **10**, 12.8 (3.0, 2.0) **11**.

Method B: with $K_3[Fe(CN)_6]$. To a stirred soln. of the appropriate dihydroxyxanthones **12**–**14** (315 mg, 1.4 mmol) and **6** (250 mg, 1.4 mmol) in Me_2CO/H_2O 1:1 (60 ml), AcONa (0.9 g, 11 mmol, in 25 ml of H_2O) and then $K_3[Fe(CN)_6]$ soln. (1.0 g, 30 mmol in 25 ml of H_2O) were added at r.t. After the reaction, the mixture was slightly acidified with 10% HCl and diluted with H_2O. The product was extracted with CH_2Cl_2; the extract was washed with H_2O, dried, and the solvent was evaporated to dryness. The crude product was analyzed by HPLC. The isolation of the components, was then carried out by prep. TLC ($CHCl_3$/MeOH 95:5). HPLC: t_R (% yield): 17.2 (29) **7**, 20.2 (11) **8**, 4.9 (27) **9**, 8.4 (0.8) **10**, 12.8 (4.0) **11**.

Conversion of (±)-cis-*Kielcorin C* (**8**) *to* (±)-trans-*Kielcorin C* (**7**). To the mixture of **7** and **8** obtained with $K_3[Fe(CN)_6]$ as described above (409.6 mg), H_2O (200 ml) was added, and the suspension was alkalinized with K_2CO_3. The suspension was stirred at r.t. for 1 h, until conversion of *cis*-isomer **8** to the *trans*-isomer **7** was complete. The mixture was extracted with AcOEt, washed with H_2O, dried (Na_2SO_4), filtered, and concentrated in vacuum to reveal a white solid (407.2 mg, 99%).

(±)-(2R,3R*)- and (±)-(2S*,3S*)-2,3-Dihydro-3-(4-hydroxy-3-methoxyphenyl)-2-(hydroxymethyl)-7H-1,4-dioxino[2,3-c]xanthen-7-one* (*(±)-trans-kielcorin C*; **7**). M.p. 243–247 ° (Me_2CO). UV (EtOH): 309, 283, 239, 204. IR (KBr): 3398, 1642, 1605, 1562, 1449, 1338, 1264, 1025, 861. ^{1}H-NMR (300.13 MHz, (D_6)DMSO): 9.25 (*s*, HO–C(4')); 8.20 (*dd*, *J* = 7.7, 1.6, H–C(8)); 7.87 (*ddd*, *J* = 8.3, 7.4, 1.6, H–C(10)); 7.70 (*d*, *J* = 8.8, H–C(6)); 7.69 (*d*, *J* = 8.3, H–C(11)); 7.49 (*ddd*, *J* = 7.7, 7.4, 0.7, H–C(9)); 7.07 (*d*, *J* = 8.8, H–C(5)); 7.07 (*d*, *J* = 1.7, H–C(2')); 6.92 (*dd*, *J* = 8.1, 1.7, H–C(6')); 6.83 (*d*, *J* = 8.1, H–C(5')); 5.15 (*d*, *J* = 7.9, H–C(3)); 5.13 (*t*, *J* = 6.1, CH_2OH); 4.36–4.40 (*m*, H–C(2)); 3.79 (*s*, MeO); 3.71–3.76 (*m*, CH_2OH); 3.40–3.50 (*m*, CH_2OH). ^{13}C-NMR (75.47 MHz, (D_6)DMSO): 175.1 (C(7)); 155.4 (C(11a)); 148.8 (C(4a)); 147.7 (C(3')); 147.2 (C(4')); 145.9 (C(12a)); 135.2 (C(10)); 131.8 (C(12b)); 126.7 (C(1')); 126.0 (C(8)); 124.5 (C(9)); 121.0 (C(7a)); 120.7 (C(6')); 118.1 (C(11)); 117.5 (C(6)); 115.7 (C(6a)); 115.4 (C(5')); 113.9 (C(5)); 111.8 (C(2')); 78.0 (C(2)); 76.5 (C(3)); 59.9 (CH_2OH); 55.7 (MeO). EI-MS: 406 (6, M^+), 356 (26), 341 (22), 327 (17), 306 (18), 292 (12), 281

(100), 261 (38), 253 (5), 228 (13), 215 (8), 180 (10), 165 (8), 141 (10), 115 (18), 105 (9), 91 (17), 77 (18), 63 (10), 55 (13). Anal. calc. for $C_{23}H_{19}O_7$: 407.1131; found: 407.1130.

(±)-(2S,3R*)- and (±)-(2R*,3S*)-2,3-Dihydro-3-(4-hydroxy-3-methoxyphenyl)-2-(hydroxymethyl)-7*H*-1,4-dioxino[2,3-c]xanthen-7-one* ((±)-cis-*Kielcorin C*; **8**). M.p. 200–202° (Me_2CO). UV (EtOH): 308, 283, 239, 204. IR (KBr): 3420, 1644, 1607, 1511, 1454, 1379, 1267, 1100, 860. ^{1}H-NMR (300.13 MHz, (D_6)DMSO): 9.09 (*s*, HO–C(4′)); 8.21 (*dd*, *J* = 7.6, 1.6, H–C(8)); 7.83 (*ddd*, *J* = 8.0, 7.5, 1.6, H–C(10)); 7.75 (*d*, *J* = 8.7, H–C(6)); 7.67 (*d*, *J* = 8.0, H–C(11)); 7.46 (*dd*, *J* = 7.6, 7.5, H–C(9)); 7.05 (*d*, *J* = 8.8, H–C(5)); 6.99 (br. *s*, H–C(2′)); 6.95–6.74 (*m*, H–C(5′), and H–C(6′)); 5.46 (*d*, *J* = 2.8, H–C(3)); 4.98 (*t*, *J* = 5.4, CH_2OH); 4.63–4.68 (*m*, H–C(2)); 3.83 (*s*, MeO); 3.42–3.61 (*m*, CH_2OH). ^{13}C-NMR (75.47 MHz, (D_6)DMSO): 175.5 (C(7)); 155.9 (C(11a)); 148.7 (C(4a)); 147.8 (C(3′)); 147.1 (C(4′)); 146.7 (C(12a)); 135.2 (C(10)); 131.1 (C(12b)); 126.7 (C(1′)); 126.4 (C(8)); 124.6 (C(9)); 121.5 (C(7a)); 119.5 (C(6′)); 118.4 (C(11)); 118.2 (C(6)); 116.4 (C(6a)); 115.9 (C(5′)); 114.1 (C(5)); 111.4 (C(2′)); 77.8 (C(2)); 76.4 (C(3)); 58.6 (CH_2OH); 56.0 (MeO). EI-MS: 406 (19, M^+), 281 (1.8), 246 (24), 184 (14), 153 (100), 149 (8), 137 (17), 111 (1.3), 93 (60), 83 (22), 72 (8), 65 (38), 59 (14), 57 (41). Anal. calc. for $C_{23}H_{19}O_7$: 407.1131; found: 407.1131.

(±)-(2R,3R*)- and (±)-(2S*,3S*)-2,3-Dihydro-3-(4-hydroxy-3-methoxyphenyl)-2-(hydroxyphenyl)-12*H*-1,4-dioxino[2,3-a]xanthen-12-one* ((±)-trans-*Kielcorin D*; **9**). M.p. 258–260° (Me_2CO). UV (EtOH): 372, 278, 259, 239, 237, 212. IR (KBr): 3498, 1635, 1602, 1309, 1270, 1228, 1047, 762. ^{1}H-NMR (300.13 MHz, (D_6)DMSO): 9.20 (*s*, HO–C(4′)); 8.04 (*dd*, *J* = 7.9, 1.6, H–C(11)); 7.80 (*ddd*, *J* = 8.1, 7.8, 1.6, H–C(9)); 7.56 (*d*, *J* = 8.1, H–C(8)); 7.45 (*d*, *J* = 9.1, H–C(5)); 7.40 (*dd*, *J* = 7.9, 7.8, H–C(10)); 7.14 (*d*, *J* = 9.1, H–C(6)); 7.10 (*d*, *J* = 1.7, H–C(2′)); 6.92 (*dd*, *J* = 8.1, 1.7, H–C(6′)); 6.83 (*d*, *J* = 8.1, H–C(5′)); 5.05 (*d*, *J* = 7.6, H–C(3)); 5.05 (*t*, *J* = 7.6, CH_2OH); 4.20–4.23 (*m*, H–C(2)); 3.78 (*s*, MeO); 3.57–3.62 (*m*, CH_2OH); 3.41 (under H_2O signal, CH_2OH). ^{13}C-NMR (75.47 MHz, (D_6)DMSO): 174.9 (C(12)); 154.6 (C(7a)); 150.9 (C(6a)); 147.6 (C(3′)); 147.0 (C(4′)); 143.1 (C(12b)); 138.9 (C(4a)); 134.8 (C(9)); 127.1 (C(1′)); 125.9 (C(11)); 124.0 (C(10)); 123.7 (C(5)); 121.7 (C(11a)); 120.5 (C(6′)); 117.6 (C(8)); 115.3 (C(5′)); 111.92, 111.86 (C(12a), C(2′)); 109.5 (C(6)); 77.2 (C(2)); 76.1 (C(3)); 60.0 (CH_2OH); 55.7 (MeO). EI-MS: 406 (6, M^+), 369 (2), 327 (7), 285 (6), 228 (14), 184 (22), 153 (100), 137 (10), 125 (13), 110 (9), 93 (61), 81 (9), 65 (35). Anal. calc. for $C_{23}H_{19}O_7$: 407.1131; found: 407.1130.

(±)-(2R,3R*)- and (±)-(2S*,3S*)-2,3-Dihydro-2-(4-hydroxy-3-methoxyphenyl)-3-(hydroxymethyl)-12*H*-1,4-dioxino[2,3-a]xanthen-12-one* ((±)-trans-*Isokielcorin D*; **10**). M.p. 136–139° (Me_2CO). UV (EtOH): 374, 280, 260, 240, 209. IR (KBr): 3436, 1626, 1520, 1459, 1383, 1319, 1269, 1110, 1060, 1028, 809. ^{1}H-NMR (500.13 MHz, (D_6)DMSO): 9.20 (*s*, HO–C(4′)); 8.12 (*dd*, *J* = 7.8, 1.4, H–C(11)); 7.81 (*ddd*, *J* = 8.1, 7.4, 1.4, H–C(9)); 7.57 (*d*, *J* = 8.1, H–C(8)); 7.43 (*d*, *J* = 9.1, H–C(5)); 7.43 (*dd*, *J* = 7.8, 7.4, H–C(10)); 7.12 (*d*, *J* = 9.1, H–C(6)); 7.04 (*d*, *J* = 1.4, H–C(2′)); 6.89 (*d*, *J* = 8.1, 1.4, H–C(6′)); 6.80 (*d*, *J* = 8.1, H–C(5′)); 5.00 (*d*, *J* = 7.8, H–C(2)); 4.99 (*t*, *J* = 7.6, CH_2OH); 4.31–4.34 (*m*, H–C(3)); 3.77 (*s*, MeO); 3.71–3.73 (*m*, CH_2OH); 3.41 (under H_2O signal, CH_2OH). ^{13}C-NMR (75.47 MHz, (D_6)DMSO): 174.6 (C(12)); 154.5 (C(7a)); 150.0 (C(6a)); 147.7 (C(3′)); 147.3 (C(4′)); 143.2 (C(12b)); 139.2 (C(4a)); 135.0 (C(9)); 127.3 (C(1′)); 126.2 (C(11)); 123.8 (C(5)); 123.7 (C(10)); 122.3 (C(11a)); 120.8 (C(6′)); 117.7 (C(8)); 115.1 (C(5′)); 111.7 (C(12a), C(2′)); 107.9 (C(6)); 78.0 (C(3)); 75.2 (C(2)); 59.4 (CH_2OH); 55.4 (MeO). EI-MS: 406 (50, M^+), 239 (7), 228 (100), 199 (25), 180 (73), 170 (11), 153 (18), 137 (64), 124 (44), 103 (5), 91 (16), 77 (14), 65 (10). Anal. calc. for $C_{23}H_{19}O_7$: 407.1131; found: 407.1131.

(±)-(2R,3S*)- and (±)-(2S*,3R)-2,3-Dihydro-3-(4-hydroxy-3-methoxyphenyl)-2-(hydroxymethyl)-11*H*-1,4-dioxino[2,3-b]xanthen-11-one* ((±)-trans-*Kielcorin E*; **11**). M.p. 247–249° (Me_2CO). UV (EtOH): 353, 307, 277, 241. IR (KBr): 3498, 1635, 1602, 1309, 1270, 1228, 1047, 762. ^{1}H-NMR (300.13 MHz, (D_6)DMSO): 9.25 (*s*, HO–C(4′)); 8.17 (*dd*, *J* = 7.7, 1.2, H–C(10)); 7.84 (*ddd*, *J* = 8.0, 7.6, 1.2, H–C(8)); 7.62 (*d*, *J* = 8.0, H–C(7)); 7.61 (*s*, H–C(12)); 7.46 (*ddd*, *J* = 7.7, 7.5, H–C(9)); 7.23 (*s*, H–C(5)); 7.06 (*d*, *J* = 1.3, H–C(2′)); 6.90 (*dd*, *J* = 8.1, 1.3, H–C(6′)); 6.82 (*d*, *J* = 8.1, H–C(5′)); 5.13 (*d*, *J* = 8.1, H–C(3)); 5.06 (br. *s*, CH_2OH); 4.33–4.35 (*m*, H–C(2)); 3.78 (*s*, MeO); 3.58 (br. *d*, *J* = 11.4, CH_2OH); 3.38 (under H_2O signal, CH_2OH). ^{13}C-NMR (75.47 MHz, (D_6)DMSO): 174.8 (C(11)); 155.7 (C(6a)); 151.1 (C(4a)); 150.6 (C(5a)); 147.7 (C(3′)); 147.3 (C(4′)); 141.3 (C(12a)); 135.0 (C(8)); 126.5 (C(1′)); 125.8 (C(10)); 124.1 (C(9)); 120.8 (C(6′)); 120.6 (C(10a)); 118.0 (C(7)); 115.4 (C(5′), C(11a)); 111.9 (C(2′)); 111.2 (C(12)); 104.8 (C(5)); 77.8 (C(2)); 77.0 (C(3)); 60.0 (CH_2OH); 55.7 (MeO). EI-MS: 406 (0.02, M^+), 369 (0.8), 327 (6), 310 (0.8), 285 (6), 267 (0.6), 260 (1.4), 246 (1), 215 (2), 208 (0.6), 184 (21), 153 (100), 137 (3), 125 (12), 110 (9), 93 (62), 81 (8), 65 (35). Anal. calc. for $C_{23}H_{19}O_7$: 407.1131; found: 407.1131.

3. *Synthesis of Xanthones* **12**–**14**. The following compounds were obtained according to the procedures cited below.

*3,4-Dihydroxy-9*H*-xanthen-9-one* (**12**) [14]. M.p. >330° $CHCl_3$ ([16]: 240–241° ($EtOH/H_2O$); [17]: 238–240° (MeOH)). UV, IR, and EI-MS: in accord with those in [17]. The analysis of the ^{1}H, ^{13}C, COSY, HETCOR,

and HMBC spectra of **12** permits us to correct the literature [17] assignment of the ^{1}H resonances of H–C(6) and H–C(5) to 7.81 (*ddd*, J = 8.6, 6.9, 1.7, H–C(6)) and 7.62 (*dd*, J = 8.6, and 0.9, H–C(5)).

*1,2-Dihydroxy-9*H*-xanthen-9-one* (**13**) [14][18]. M.p. 163–165° (CH_2Cl_2/hexane); [19]: 166–167° (EtOH/H_2O)). UV (EtOH): 261, 243, 232, 203 (33300), 28800, 14200, 11000); UV (EtOH + NaOH): 311, 236, 222 (76000, 23900, 17000). UV (EtOH + $AlCl_3$): 305, 283, 266, 204 (33000, 22900, 8700). IR (KBr): 3467, 1606, 1475, 1365, 1288, 1045, 748. ^{1}H-NMR (300.13 MHz, (D_6)DMSO): [20] 12.45 (*s*, HO–C(1)); 9.42 (*s*, HO–C(2)); 8.16 (*dd*, J = 7.9, 1.6, H–C(8)); 7.81 (*ddd*, J = 8.4, 7.0, 1.6, H–C(6)); 7.60 (*dd*, J = 8.4, 0.8, H–C(5)); 7.46 (*ddd*, J = 7.9, 7.0, 0.8, H–C(7)); 7.32 (*d*, J = 9.0, H–C(4)); 6.96 (*d*, J = 9.0, H–C(3)). ^{13}C-NMR (75.47 MHz, (D_6)DMSO): 182.3 (C(9)); 155.9 (C(4b)); 148.3 (C(4a)); 147.6 (C(1)); 140.2 (C(2)); 136.4 (C(6)); 125.4 (C(8)); 124.5 (C(3)); 124.2 (C(7)); 119.3 (C(8a)); 118.0 (C(5)); 108.8 (C(9a)); 106.2 (C(4)). EI-MS: 230 (1, $[M + 2]^+$), 229 (1, $[M + 1]$), 228 (70, M^+), 199 (13), 126 (5), 115 (4), 78 (95), 63 (100).

*2,3-Dihydroxy-9*H*-xanthen-9-one* (**14**) [14][17][21]. M.p. >330° (Me_2CO/hexane) ([14]: >350° (AcOEt); [17]: 293–295° ($CHCl_3$/MeOH)). UV, IR and EI-MS: in accord with those in [17]. The unequivocal assignments of ^{13}C resonances, mainly on the basis of 1D selective INEPT and 2D HETCOR experiments, allowed us to correct some ^{1}H and ^{13}C resonances of **14** [17], namely the assignment of the ^{1}H resonances of H–C(6) to 7.78 (*ddd*, J = 8.4, 6.8, 1.7) and H–C(5) to 7.58 (*d*, J = 8.4) and the ^{13}C resonances of C(2) (144.1), C(3) (151.3), and C(4a) (154.2).

Methyl 2-Bromobenzoate (**15**) [22], *Methyl 2-(3,4-Dimethoxyphenoxy)benzoate* (**17**) [11–13], *2-(3,4-Dimethoxyphenoxy)benzoic Acid* (**18**) [11][12], *and 1,2-Dimethoxy-9*H*-xanthen-9-one* (**19**) [12][24]: Data identical to those reported in [12][25].

*2,3-Dimethoxy-9*H*-xanthen-9-one* (**20**) [13][25]. M.p. 170–171° (CH_2Cl_2/pentane) ([26]: 164–165° (Me_2CO); [17]: 157–158° ($CHCl_3$); [27]: 155–159° (CH_2Cl_2/heptane); [28]: 165–167° ($CHCl_3$/petroleum ether)). IR and EI-MS: in accord with those in [17]. The unequivocal assignments of ^{13}C resonances, mainly on the basis of 1D selective INEPT and 2D HETCOR experiments, allowed us to correct some ^{1}H and ^{13}C resonances of **20** [29]. ^{1}H-NMR (200.13 MHz, (D_6)DMSO): 8.16 (*dd*, J = 7.9, 1.5, H–C(8)); 7.82 (*ddd*, J = 8.4, 7.4, 1.5, H–C(6)); 7.60 (*d*, J = 8.4, H–C(5)); 7.49 (*s*, H–C(1)); 7.44 (*ddd*, J = 7.9, 7.4, 0.9, H–C(7)); 7.20 (*s*, H–C(4)); 3.94 (*s*, MeO–C(3)); 3.87 (*s*, MeO–C(2)). ^{13}C-NMR (50.03 MHz, (D_6)DMSO): 174.8 (C(9)); 155.7 (C(4b)); 155.6 (C(4a)); 152.0 (C(3)); 146.7 (C(2)); 134.8 (C(6)); 125.9 (C(8)); 124.3 (C(7)); 120.9 (C(8a)); 118.0 (C(5)); 114.0 (C(9a)); 104.7 (C(1)); 100.5 (C(4)); 56.6 (*Me*O–C(3)); 55.9 (*Me*O–C(2)).

4. *Cell-Growth Assay.* The effects of compounds on the growth of human cancer cell lines were evaluated according to the procedure adopted in the NCI's *in vitro* anticancer drug screening that uses the SRB assay to assess growth inhibition [30]. This colorimetric assay estimates cell number indirectly by staining cellular protein with the protein-binding dye SRB. Three human cancer cell lines were used, MCF-7 (breast cancer), TK-10 (renal cancer), UACC-62 (melanoma). Cells were routinely maintained as adherent cell cultures in *RPMI-1640* medium containing 5% heat-inactivated fetal bovine serum, 2 mM glutamine, and 50 µg/ml gentamicin at 37° in a humidified air incubator containing 5% CO_2. For the SRB assay, each cell line was plated at an appropriate density (1.5×10^5 cells/ml to MCF-7 and TK-10, 1.0×10^5 cells/ml to UACC-62) in 96-well plates and allowed to attach overnight. Cells were then exposed for 48 h to serial concentrations of compounds and to the positive control doxorubicin. Following this incubation period, the adherent cells were fixed *in situ*, and washed and dyed with SRB. The bound stain was solubilized, and the absorbance was measured at 492 nm in a microplate reader. Growth inhibition of 50% (GI_{50}) as well as toxicity of compounds was determined as described in [31]. Toxicity of xanthone derivatives was inferred from the SRB assay by comparing, after 48 h, the absorbance of the wells with xanthone derivative-treated cells with the absorbance of the wells containing untreated cells that were fixed at time zero (time at which compounds were added). Lower absorbances after 48 h of treatment indicate occurrence of cell death instead of growth arrest.

5. *Lymphocyte Assays.* The effects of compounds on the mitogenic response of human lymphocytes to PHA (10 µg/ml) were evaluated with a modified colorimetric MTT assay [32]. This assay was previously described by us [33]. Mononuclear cells were isolated from heparinized peripheral blood of healthy volunteers by *Histopaque-1077* density centrifugation. The toxicity of compounds against human lymphocytes was evaluated by an assay based on the ability of viable cells to reduce the colourless tetrazolium salt MTT to a colored formazan product. Briefly, in flat-bottom 96-well plates, $2-3 \times 10^6$ mononuclear cells/ml in *RPMI-1640* medium containing 10% heat-inactivated foetal bovine serum, 2 mM glutamine, and 50 µg/ml of gentamicin were exposed for 24 h to the various concentrations of each compound. Following this incubation period, the MTT soln. (1 mg/ml) was added. After incubation for 4 h, the MTT formazan products were solubilized with SDS/DMF soln. (20% SDS in a 50% soln. of DMF, pH 4.7) overnight at 37°. Absorbance (OD 550 nm) of the colored soln. was measured with a plate reader. Lymphocytoxicity, determined in terms of the percentage of

viable cells, was present when the viability of the exposed cells was less than 70% of the nonexposed control cells.

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ANEXO III

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Quantitative Analysis of Kielcorins in Biomimetic Synthesis by Liquid Chromatography/UV Detection

M. Emília Sousa,[1,2] Carlos M. M. Afonso,[1,*] and Madalena M. M. Pinto[1]

[1]Centro de Estudos de Química Orgânica, Fitoquímica e Farmacologia da Universidade do Porto, Faculdade de Farmácia, Porto, Portugal
[2]Instituto Superior Ciências Saúde/Norte, Gandra, Portugal

ABSTRACT

An HPLC/UV assay was developed for separation and quantification of isomeric kielcorins in different synthetic crude products. *trans*-(±)-Kielcorin C, *cis*-(±)-kielcorin C, *trans*-(±)-kielcorin D, *trans*-(±)-isokielcorin D, and *trans*-(±)-kielcorin E were obtained by oxidative coupling of coniferyl alcohol and an appropriate xanthonic building block, 3,4-, 1,2-, and 2,3-dihydroxyxanthone, respectively, with silver carbonate, silver oxide, and potassium hexacyanoferrate (III). The isomeric kielcorins were separated and quantified directly in the nine crude

*Correspondence: Carlos M. M. Afonso, Centro de Estudos de Química Orgânica, Fitoquímica e Farmacologia da Universidade do Porto, Faculdade de Farmácia, Rua Aníbal Cunha, 164 4050/047, Porto, Portugal; E-mail: cafonso@ff.up.pt.

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products obtained. The assay was validated with respect to specificity, linearity, range, limits of quantification, and detection.

Key Words: Liquid chromatography; Isomeric separation; Kielcorins; Oxidative coupling.

INTRODUCTION

Kielcorins are 1,4-benzodioxane derivatives belonging to the xanthonolignoids, with a skeleton in which a phenylpropane unit is linked to a xanthone nucleus through a dioxane bridge.[1] We have previously achieved a biomimetic synthesis of natural kielcorin and kielcorin B and evaluated their biological activities.[2,3] These compounds exhibited an interesting hepatoprotective activity against *tert*-butylhydroperoxide-induced toxicity in isolated rat hepatocytes.[4] In order to obtain compounds belonging to the kielcorin's family for structure/activity relationship studies, we have synthesized *trans*-(±)-kielcorin C (1), *cis*-(±)-kielcorin C (2), *trans*-(±)-kielcorin D (3), *trans*-(±)-isokielcorin D (4), and *trans*-(±)-kielcorin E (5).

Prior work on the synthesis of 1,4-benzodioxans showed the influence of several oxidising agents in the nature and amounts of the products formed in oxidative coupling type reactions.[5–7] Concerning biomimetic synthesis of xanthonolignoids, little information has been accumulated,[2,3,8] hence, the importance of investigating the oxidative coupling reaction in the presence of several oxidizing agents. The synthetic approach of these constitutional isomers was based on biomimetic pathway by oxidative coupling of coniferyl alcohol[6] with an appropriate xanthone:[2,3] 3,4-dihydroxy (7), 1,2-dihydroxy (8), and 2,3-dihydroxyxanthone (9), for C, D, and E series, respectively (Figure 1), using different oxidising agents: silver carbonate (Ag_2CO_3), silver oxide (Ag_2O), and potassium hexacyanoferrate (III) ($K_3[Fe(CN)_6]$).

Attempts to resolve the isomeric mixtures of kielcorins 1-2 and 3-4 by preparative thin layer chromatography were unsuccessful in separating *trans*/*cis* or normal/iso forms, respectively, in a quantitative mode. Hence, we considered developing a method for isomeric resolution and quantification of kielcorins 1–5 that allow us to compare the results obtained from different synthetic conditions.

In recent years, high-liquid chromatography in reversed-phase mode (RPHPLC) has been applied successfully to a number of isomeric separations due to the selectivity and sensitivity of this chromatographic technique and to faster separations of positional[9–11] and stereoisomers.[12,13] Although, there is no data concerning the application of HPLC to xanthonolignoids,

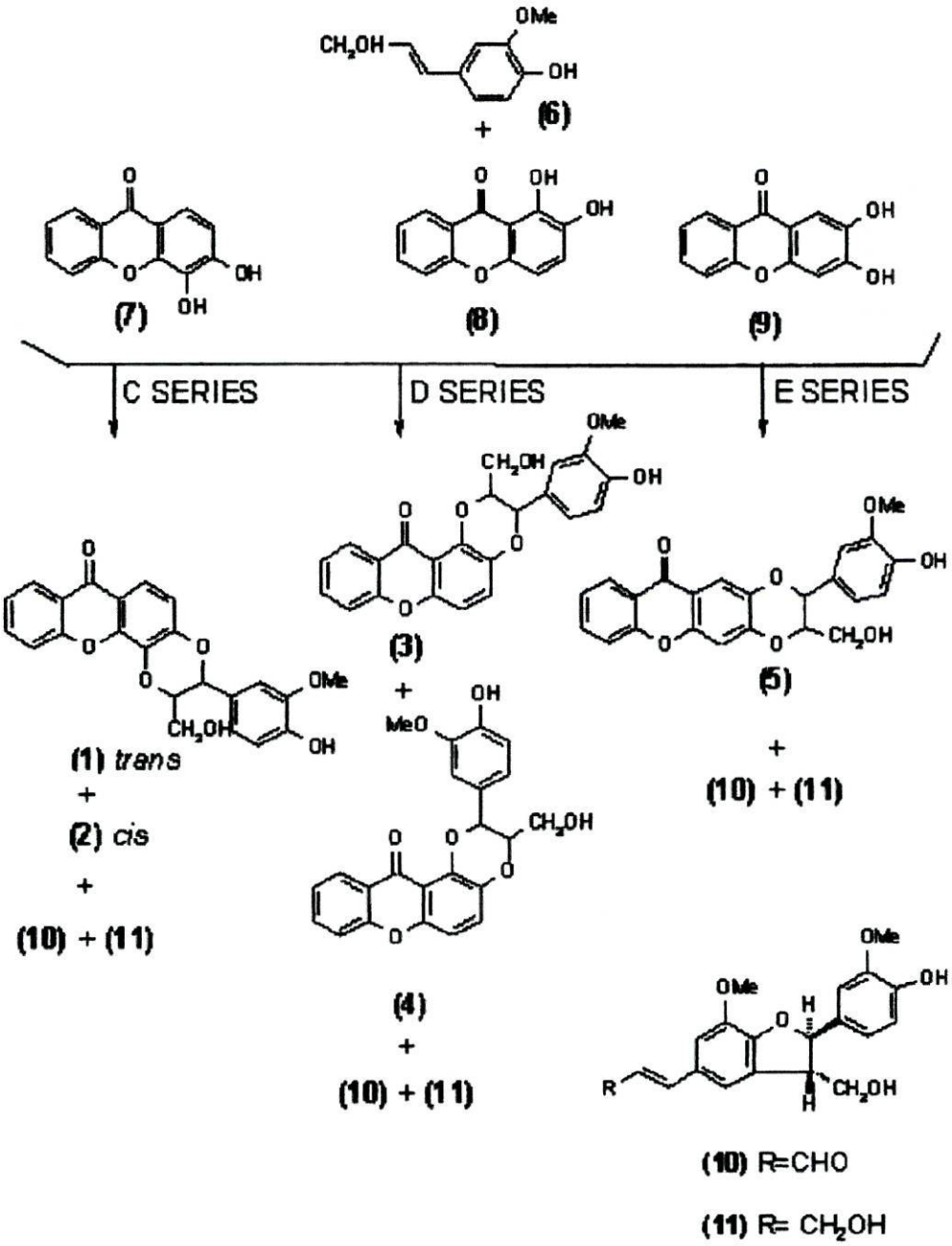


Figure 1. One-step biomimetic synthesis of kielcorins: *trans*-(±)-kielcorin C (1), *cis*-(±)-kielcorin C (2), *trans*-(±)-kielcorin D (3), *trans*-(±)-isokielcorin D (4), and *trans*-(±)-kielcorin E (5).

this technique has been used to separate compounds with similar features, namely xanthones[14–16] and 1,4-benzodioxane derivatives.[5]

In this work, we present the development and validation of an RPHPLC method with UV detection that allows the direct quantification of isomeric kielcorins from the crude product. This method was applied to the analysis of the isomeric kielcorins obtained in nine crude products from a comparative

study using different oxidising agents (silver carbonate, silver oxide, and potassium hexacyanoferrate (III)).

EXPERIMENTAL

Instrumental and Operating Conditions

A model 880 PU (Jasco, Japan) equipped with a 875/UV detector (Jasco, Japan), a Rheodyne 7125 injector, and a CSW 1.7 integrator was employed. Analysis was performed at 25°C on a C 18 Nucleosil column (5 μm, 250 × 4.6 mm I.D.), from Macherey-Nagel (Düren, Germany) equipped with a Macherey-Nagel guard precolumn. Separations of the crude products were performed in isocratic work at a flow rate of 1 mL/min with detection on 239 nm for C and D series, and 241 nm for E series. The sample injection volume was 20 μL. For compounds 1–2, separation was achieved with methanol/1% acetic acid in water (6 : 4 *v*/*v*), for compounds 3–4, with acetonitrile/1% acetic acid in water (5 : 5 *v*/*v*), and for compound 5, with methanol/1% acetic acid in water (7 : 3 *v*/*v*). The column void time (t_0) was considered to be equal to the peak of the solvent front and was taken from each particular run.

Reagents and Materials

Silver carbonate, sodium acetate, and potassium hexacyanoferrate (III) were supplied by Merck (Darmstadt, Germany). Coniferyl alcohol and silver oxide were obtained from Sigma (Steunheim, Germany). Acetone, toluene, and acetic acid glacial 100% were of analytical grade from Merck. Methanol and acetonitrile were of HPLC grade from Merck. HPLC ultrapure water was generated by a Milli-Q system (Millipore, Bedford, MA, USA). The mobile phases were degassed for 15 min in an ultrasonic bath before use. All samples were filtered through a hydrophilic Durapore-GV membrane of 0.45 μm pore size from Millipore before injection.

General Procedure for the Synthesis of Kielcorin Derivatives

With Silver Carbonate/Silver Oxide

The appropriate dihydroxyxanthone (7–9) (315 mg, 1.4 mmol) and coniferyl alcohol (6) (250 mg, 1.4 mmol) were taken in toluene/acetone (1 : 1) and stirred with 2.0 mmol of the oxidizing agent (550 mg of silver carbonate/460 mg of

silver oxide) in the dark for 2–3 days. The suspension was filtered, the filtrate evaporated, and the crude product was analysed by HPLC.

With Potassium Hexacyanoferrate (III)

To a stirred solution of the appropriate dihydroxyxanthone (7–9) (315 mg, 1.4 mmol) and coniferyl alcohol (6) (250 mg, 1.4 mmol) in acetone/water (1 : 1), sodium acetate (0.9 g, 11 mmol, in 25 mL of water), and then potassium hexacyanoferrate (III) solution (1.0 g, 30 mmol in 25 mL of water) was added at room temp. After reaction, the mixture was slightly acidified with 10% hydrochloric acid and diluted with water. The product was extracted with dichloromethane; the extract was washed with water, dried, and the solvent evaporated to dryness. The crude product was analyzed by HPLC.

Sample Preparation

The nine crude products obtained (ca. 600 mg) were accurately weighed and dissolved in 500 mL of methanol. Serial dilutions in methanol were made to the following final concentrations: 100, 200, and 300 μg/mL, for use in determination of precision assays.

Stock standard solutions of each kielcorin 1–5 were prepared by dissolving 2.5 mg accurately weighed in 25 mL of methanol. Standard solutions were obtained by the dilution of stock solutions with methanol at least to five different concentrations over the range of interest, in this case, 5.0, 25, 50, 75, 100, and 200 μg/mL for *trans*-kielcorin C (1), 0.5, 2.5, 3.75, 12.5, and 50 μg/mL for *cis*-kielcorin C (2), 10, 25, 50, 75, and 100 μg/mL for *trans*-kielcorin D (3), 0.25, 0.5, 1.0, 2.5, 5.0, 10, and 25 μg/mL for *trans*-isokielcorin D (4), and 0.5, 1.0, 2.5, 5.0, and 25 μg/mL for *trans*-kielcorin E (5).

RESULTS AND DISCUSSION

Method Development

The present assay was developed for the analysis of three series of kielcorins: C series (1–2), D series (3–4), and E series (5) (Fig. 1). The separations of the kielcorin isomers were performed on RPHPLC and were optimised for each series to the described experimental conditions used for the validation procedure (Figs. 2 and 3). Chromatographic factors are listed in Table 1. Several kinds of mobile phase compositions were investigated,

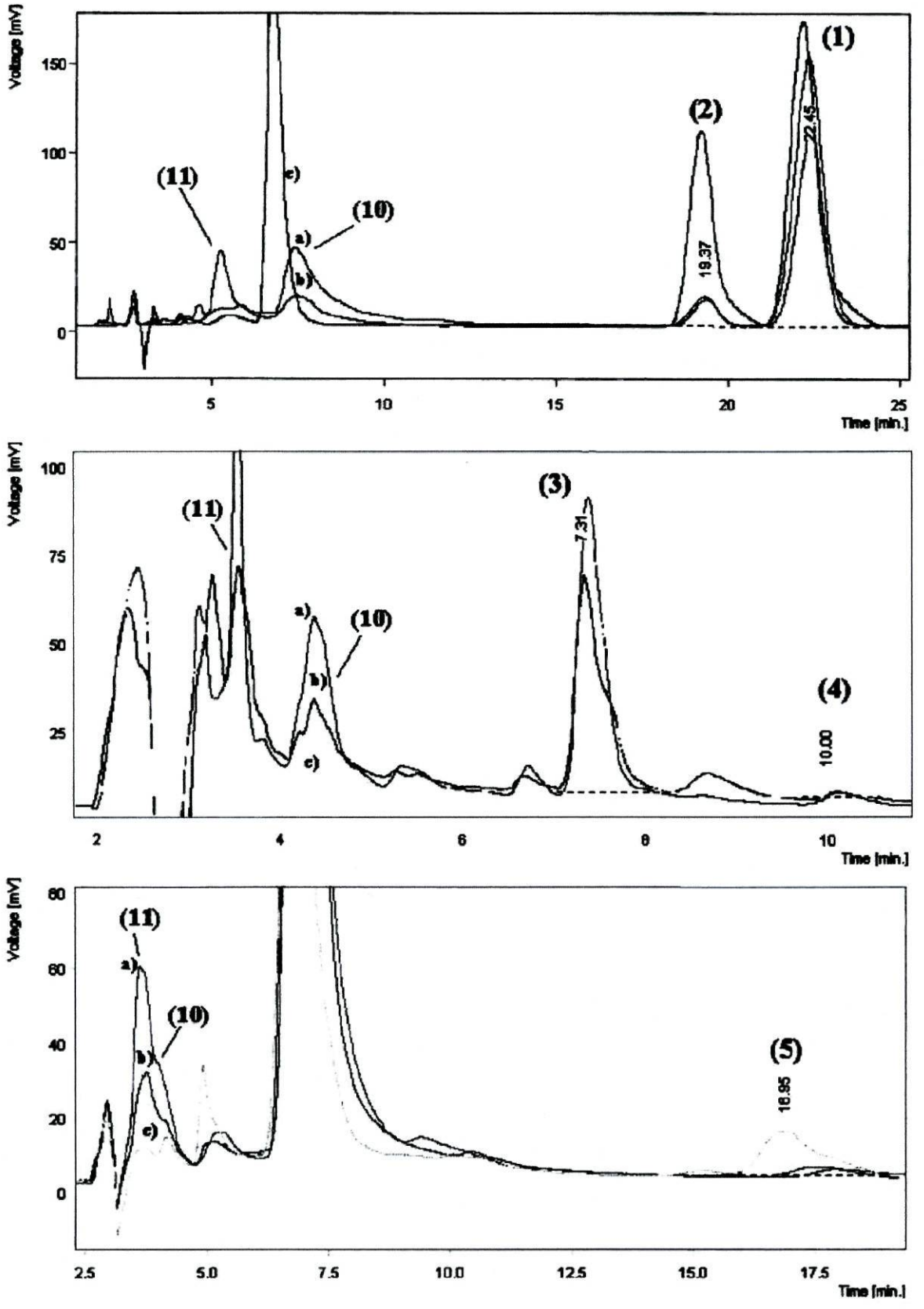


Figure 2. Chromatograms of 200 g/mL solutions of the crude products obtained in C, D, and E series using as oxidizing agent: a) Ag_2CO_3, b) Ag_2O, and c) $K_3[Fe(CN)_6]$.

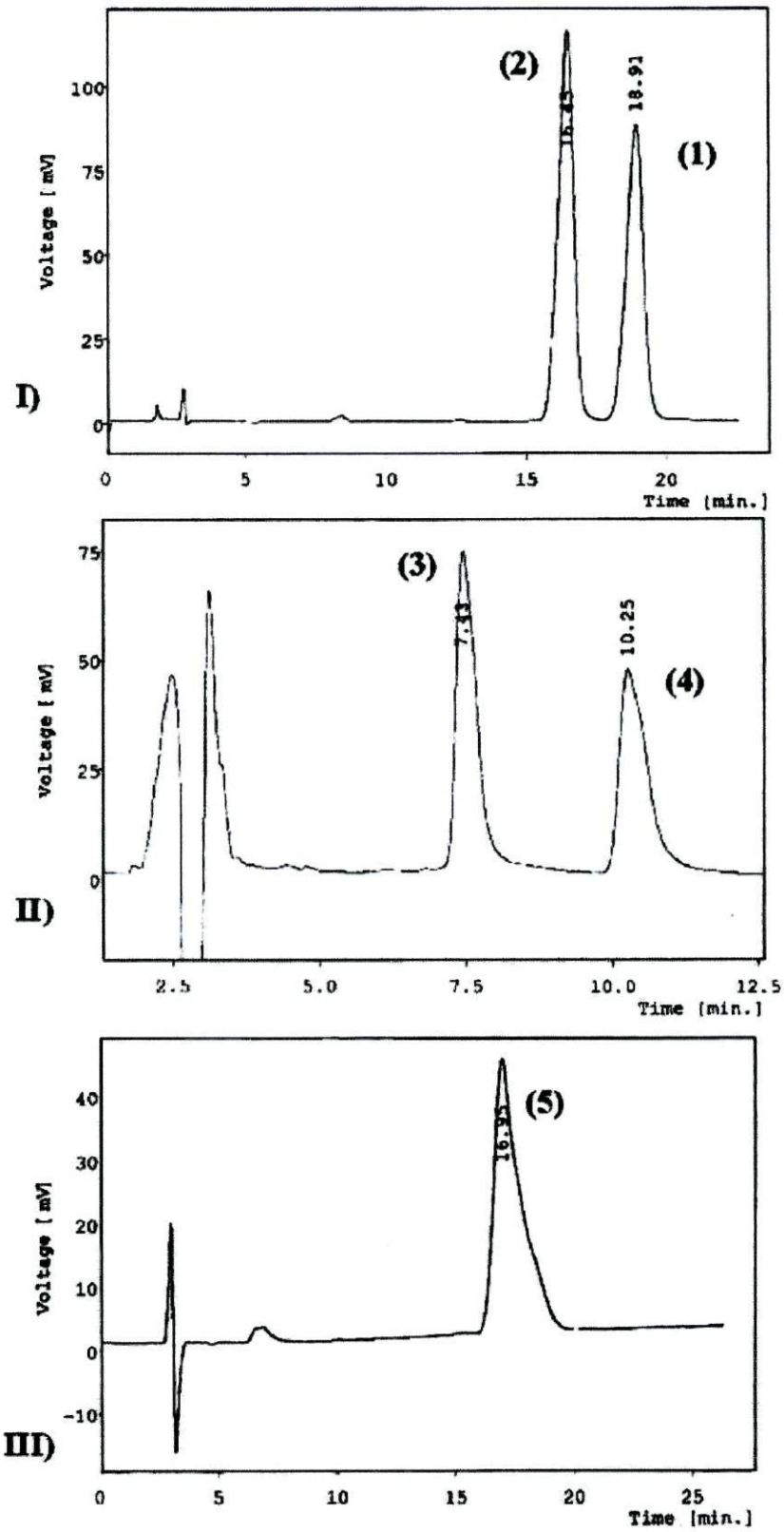


Figure 3. Chromatograms of standards//conditions: I) trans-(±)-kielcorin C (1) and cis-(±)-kielcorin C (2)// methanol/1% acetic acid in water (6 : 4), II) trans-(±)-kielcorin D (3) and trans-(±)-isokielcorin D (4)// acetonitrile/1% acetic acid in water (5 : 5), and III) trans-(±)-kielcorin E (5)// methanol/1% acetic acid in water (7 : 3).

Table 1. Separation performance:[a] retention factors (k'_1), separation factors (α) and resolution factors (R_s), of the investigated kielcorin derivatives 1–5 in optimised chromatographic conditions.[b]

	Mobile phase	Oxidising agent	k'_1	α	R_s
1	MeOH/HOAc 1% in H_2O (6 : 4)	Ag_2CO_3/Ag_2O	4.3	1.2	3.5
		$K_3[Fe(CN)_6]$	4.3	1.2	3.6
2	MeOH/HOAc 1% in H_2O (6 : 4)	Ag_2CO_3/Ag_2O	3.5	4.9	12.7
		$K_3[Fe(CN)_6]$	3.5	5.6	20.4
3	MeCN/OHAc 1% in H_2O (5 : 5)	$Ag_2CO_3/Ag_2O/$ $K_3[Fe(CN)_6]$	1.2	1.1	2.2
4	MeCN/HOAc 1% in H_2O (5 : 5)	Ag_2CO_3/Ag_2O	2.1	1.3	3.3
		$K_3[Fe(CN)_6]$	2.1	1.6	3.2
5	MeOH/HOAc 1% in H_2O (7 : 3)	$Ag_2CO_3/Ag_2O/$ $K_3[Fe(CN)_6]$	3.8	2.1	7.3

[a]The values are the mean of three experiments. The column void time (t_0) was considered to be equal to the peak of the solvent front and was taken from each particular run. It was about 2.2 min in MeOH/HOAc 1% in H_2O (6 : 4), 2.5 min in MeCN/HOAc 1% in H_2O (5 : 5) and 2.6 min MeOH/HOAc 1% in H_2O (7 : 3).
[b]Chromatographic conditions as in Figure 2 (200 µg/mL solutions of the crude products).

and under optimised conditions, kielcorins 1–5 were fully resolved ($R_s > 2$) and their retention factors (k') satisfactory (1.2–4.8).[17]

The standard compounds 1–5 were previously isolated and purified from crude products and characterised by spectroscopic methods. Detailed synthesis and complete assignment will be published elsewhere.

Assay Validation

The assay was validated with respect to specificity, linearity, range, limits of quantification and detection, and precision. Accuracy was inferred once precision, linearity, and specificity had been established. The terms are defined according to ICH guideline 3AQ[18] and to U. S. Pharmacopeia 24.[19]

The specificity of the analytical method in this study was determined by the analysis of the side products of the oxidative coupling reactions,[20] phenylcoumaran (10) and dehydrodiconiferyl alcohol, (11) and the xanthonic precursors (7–9) (Figure1) in standard solutions, which absorb in the same λ values. Under the applied conditions, no interference from the components

of the crude product was observed at the retention time for kielcorins 1–5 (Fig. 2).

Linearity was evaluated in triplicates of at least five calibration standard solutions of the compounds 1–5. The range of the calibration curves was set at 80 to 120% of the kielcorins concentration in the crude product solution.[18,19] The regression line was calculated as $y = a + bx$, where x was the kielcorin concentration (μg/mL) and y the response (peak area expressed as mAU). The calibration curves were obtained using the linear least squares regression procedure. The calibration data of the kielcorin derivatives 1–5 is summarized in Table 2. The RSD (relative standard deviation) values for the response factors of three experiments were in the range 0–5% (3.6, 3.6, 4.0, 3.7, and 2.0 % for compounds 1–5, respectively), and they were considered adequate for verifying the linearity of the regression.[21] Linear relationships with regression coefficients (R) of at least 0.999 were found for all substances, hence, there were linear relationships between the amounts of kielcorins 1–5 and the detector response.

The limits of detection (LODs) and quantification (LOQs) were evaluated by analysing ten blank samples. LODs and LOQs were expressed as the standard deviation of these responses, divided by the slope and multiplied by a factor of 3.3 for LODs and 10 for LOQs.[17,18] The LODs were found to be 0.050 μg/mL, 0.051 μg/mL, 0.052 μg/mL, 0.054 μg/mL, and 0.010 μg/mL for *trans*-kielcorin C (1), *cis*-kielcorin C (2), *trans*-kielcorin D (3), *trans*-isokielcorin D (4), and *trans*-kielcorin E (5), respectively. The LOQs were found to be 0.15 μg/mL, 0.15 μg/mL, 0.16 μg/mL, 0.16 μg/mL, and 0.03 μg/mL for *trans*-kielcorin C (1), *cis*-kielcorin C (2), *trans*-kielcorin D (3), *trans*-isokielcorin D (4), and *trans*-kielcorin E (5), respectively.

The precision of the assay was investigated with respect to repeatability and intermediate precision. For intra/day precision three concentrations of the crude product were analysed within 24 hours. Within each series, every sample was injected at least five times. For intermediate precision, samples from

Table 2. Calibration data of the kielcorin derivatives 1–5.

	Range (μg/mL)	y-Intercept[a]	Slope[a]	Correlation coefficient (R)
1	5.00–200	−56.45 ± 2.03	141.4 ± 5.09	0.9991
2	0.50–50.0	93.14 ± 3.35	137.8 ± 4.96	0.9988
3	10.0–100	−76.60 ± 3.06	105.2 ± 4.21	0.9990
4	0.25–25.0	13.58 ± 0.50	129.6 ± 4.79	0.9993
5	0.50–25.0	44.99 ± 0.90	136.8 ± 2.74	0.9993

[a]The values are the mean ± standard deviation of three experiments.

three concentrations of the crude products were analysed on five consecutive days assaying, in quintuplicates. Precision was expressed as relative standard deviation (RSD) given by the standard deviation value of the peak area divided by the mean.[18] Table 3 summarizes the RSDs for repeatability and intermediate precision. The RSD values of results from replicate analyses ranged between 0.95 and 10.4% showing that the precision of the method was acceptable.

Application

The aim of our work was the analysis of kielcorin derivatives directly in crude products obtained in the oxidative coupling type reactions. To evaluate the influence of oxidizing agents in the nature and quantities of the isomeric kielcorins, we developed a rapid method for the separation of these isomeric compounds. This method was applied in a comparative study using as oxidizing agents silver carbonate, silver oxide, and potassium hexacyanoferrate (III).

Data of the quantification results are summarized in Table 4. In C series, none of the oxidizing agents used afforded stereoselectivity, although

Table 3. Precision data for HPLC-UV assay of the kielcorin derivatives 1–5.

	Concentration of the crude product (mg/mL)	Repeatability RSD (%) ($n^a = 15$)	Intermediate precision RSD (%) ($n^a = 30$)
1	0.100	10.43^b	$4.569^{c,d}$
	0.200	1.602^b	$3.153^{c,d}$
	0.300	1.418^b	$6.147^{c,d}$
2	0.100	7.768^b	$3.541^{c,d}$
	0.200	1.120^b	$3.081^{c,d}$
	0.300	1.354^b	$4.433^{c,d}$
3	0.100	2.364^c	$3.752^{b,d}$
	0.200	4.735^c	$1.336^{b,d}$
	0.300	6.216^c	$4.082^{b,d}$
4	0.100	4.500^c	$9.270^{b,d}$
	0.200	2.571^c	$2.682^{b,d}$
	0.300	7.741^c	$2.851^{b,d}$
5	0.100	1.189^c	$2.134^{b,d}$
	0.200	1.008^c	$0.846^{b,d}$
	0.300	1.020^c	$0.947^{b,d}$

[a] n = number of injections.

[b,c,d] RSD values for peak area of compounds 1–5 obtained in the oxidative coupling using Ag_2O, Ag_2CO_3, and $K_3[Fe(CN)_6]$ as oxidizing agents respectively.

Table 4. Quantification of compounds 1–5 in the crude products.[a]

	Ag_2CO_3[b]	Ag_2O[b]	$K_3[Fe(CN)_6]$[b]
1	27%	36%	39%
2	3.1%	3.5%	11%
3	22%	20%	34%
4	0.4%	0.7%	1.1%
5	2.4%	1.9%	10.4%

[a]The values are represented in yields of the oxidative coupling reactions.
[b]Oxidizing agents used in the comparative study.

when using silver carbonate and silver oxide, less amounts of the *cis* isomer 2 was obtained. Two dimeric compounds, phenylcoumaran (10) and dehydrodiconiferyl alcohol (11) were also found as secondary derivatives (Figure 2) in all the reactions.[3] By carrying out the oxidative coupling in potassium hexacyanoferrate (III), the quantities of 10 and 11 were markedly reduced and higher yields of kielcorins were obtained. The most efficient method involved the use of potassium hexacyanoferrate (III) as oxidizing agent in the oxidative coupling reaction (Table 4).

CONCLUSIONS

A method based on RPHPLC/UV has been developed for the separation of isomeric xanthonolignoids in crude products reactions. Baseline separations were successfully achieved for all kielcorin derivatives under study. The assay was found to be precise, sensitive, and calibration curves were linear within the studied concentration range, allowing the quantification of these compounds. The method was applied to a comparative study using different oxidizing agents in oxidative coupling type reactions. Further investigations, namely enzymatic oxidation and solid phase reactions, are under study in order to improve yields and regio/stereoselectivity in kielcorins synthesis. Hence, HPLC methodology can be successfully applied for direct analysis of xanthonolignoids and quantitative studies in synthetic crude products.

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ANEXO IV

Enantiomeric Resolution of Kielcorin Derivatives by HPLC on Polysaccharide Stationary Phases using Multimodal Elution

Emília P. Sousa[1,2], M. E. Tiritan[1,2], Regina V. Oliveira[3] , C. M. M. Afonso[1], Q. B. Cass[3], Madalena M. M. Pinto[1 *]

[1]Centro de Estudos de Química Orgânica, Fitoquímica e Farmacologia-Universidade do Porto; Faculdade de Farmácia, Rua Aníbal Cunha, 164, 4050-047, Porto, Portugal

[2]I.S.C.S. - Norte, Rua Central da Gandra, 1317, 4580 Gandra, Portugal

[3]Departamento de Química-Universidade Federal de São Carlos-CP676-13565-905-São Carlos-SP, Brazil

KEY WORDS: xanthonolignoids; PKC inhibitors; amylose stationary phases; cellulose stationary phases; polar organic conditions

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* Correspondence to: Madalena M. M. Pinto, Laboratório de Química Orgânica, Faculdade de Farmácia, Rua Aníbal Cunha, 164, 4050-047, Porto, Portugal. Tel./fax: +351-222057358. E-mail: madalena@ff.up.pt

ABSTRACT Analytical HPLC methods using carbamate chiral stationary phases of polysaccharides derivatives were developed for the enantiomeric resolution of five racemic mixtures of xanthonolignoids: *rac-trans*-kielcorin C, *rac-cis*-kielcorin C, *rac-trans*-kielcorin D, *rac-trans*-isokielcorin D and *rac-trans*-kielcorin E. The separations were studied with the stationary phases cellulose *tris*-3,5-dimethylphenylcarbamate, amylose *tris*-3,5-dimethylphenylcarbamate, amylose *tris*-(S)-1-phenylethylcarbamate and amylose *tris*-3,5-dimethoxyphenylcarbamate, under normal, reversed-phase and polar organic conditions. Chiral recognition of those chiral stationary phases, the influence of mobile phases upon the enantiomer separation and the effects of the solutes structural features on chiral discrimination were studied. The best performance was achieved on an amylose *tris*-3,5-dimethylphenylcarbamate. Polar organic conditions presented shorter retentions times and better resolutions, being a valuable alternative to more common alcohol-hexane and reversed-phase conditions.

The 1,4-benzodioxane ring system has aroused increasing interest due to its presence in a large number of structures of important therapeutic agents,[1-7] whose biological activity, in some cases, is considerably influenced by the chirality of the 1,4-benzodioxane moiety.[3,8-11]

Kielcorins are 1,4-benzodioxane derivatives belonging to the family of xanthonolignoids which present interesting biological activities.[12] We have recently described the synthesis of five isomers: *rac-trans*-kielcorin C (**1**), *rac-cis*-kielcorin C (**2**), *rac-trans*-kielcorin D (**3**), *rac-trans*-isokielcorin D (**4**) and *rac-trans*-kielcorin E (**5**) (Fig. 1)[13] as well as the chromatographic separation of the pairs of isomers **1/2** and **3/4**.[14] The racemates of kielcorins **1-5** demonstrated an antiproliferative effect against three human tumour cell lines, MCF-7 (breast), TK-10 (renal), and UACC-62 (melanoma)[13] and also revealed an effect compatible with PKC inhibition.[15] Therefore, the resolution of the racemates **1-5** seems advisable.

Although some enantiomeric resolution methods[9] including reverse-phase HPLC[10] were developed for chiral 1,4-benzodioxane lignans, the resolution of quiral xanthonolignoids has never been reported before.

This paper describes an investigation of the resolution of chiral xanthonolignoids **1-5** on arylcarbamate derivatives of cellulose and amylose (Fig. 2). The influence of structural features of the analytes on discrimination of enantiomers and the contribution of different substituents on the aryl groups of polysaccharide derivatives are discussed. This work also explores the influence of different mobile phases upon enantiomeric separation and illustrates the potential of using multimodal elution.

MATERIALS AND METHODS

Chemicals

All solvents were HPLC grade and purchased from Merck (Darmstadt, Germany) and ultrapure water was generated by a Milli-Q system (Millipore, Bedford, MA, USA).

The columns were prepared in the laboratory as described elsewhere[16,17] and consisted of cellulose *tris*-3,5-dimethylphenylcarbamate (CSP1), amylose *tris*-3,5-dimethylphenylcarbamate (CSP2), amylose *tris*-(S)-1-phenylethylcarbamate (CSP3) and amylose *tris*-3,5-dimethoxyphenylcarbamate (CSP4), the carbamates were coated onto APS-Nucleosil (500Å, 7μm, 20%w/w) and packed into a stainless-steel 15 x 0,46 cm ID size column.

Analytes **1-5** were isolated by chromatography and their structures, *trans* or *cis* and normal or iso forms, elucidated by spectroscopic methods.[13] The UV spectra of the isomers **1-5** were very similar. The maximum wavelengths were, respectively, 239 nm for kielcorins **1-4** and 242 nm for kielcorin **5**.

Chromatography

A System SMI Pump Series II (Gloucester, UK) equipped with a Rheodyne 7125 injector fitted with a 20μL loop, a TSP-UV6000LP detector and a Chromquest for Windows NT integrator was employed. Analyses were performed in isocratic work with a multimodal operation: the mobile phase compositions were *n*-hexane and a modifier, ethanol or 2-propanol, in normal mode, methanol/acetonitrile-trifluoroacetic acid 0.1% in water in reversed phase mode, and methanol-ethanol, 2-propanol-ethanol, acetonitrile-methanol in polar organic conditions, all in various proportions. The mobile phases were degassed for 15 min in an ultrasonic bath before use. All samples were filtered through a hydrophilic Durapore-GV membrane of 0.45 μm pore size from Millipore before injection. Chromatographic separations were performed at room temperature, at a flow rate of 0.4 ml.min^{-1}. In switching the elution mode, the column was conditioned by recycling 100% ethanol for about 6 hours at 0.4 ml.min^{-1}.

The retention factor (k) was determined as [$k = (t_R-t_0)/t_0$] where t_R is the retention time for the first eluted enantiomer. The enantioselectivity factor (α) was calculated as [$\alpha = k_2/k_1$]. The resolution factor (Rs) was determined as [$Rs = 2\ (t_{R2}-t_{R1})/\ (W_1+W_2)$] were t_{Ri} and W_i are the retention times and the base-widths of the peak of each enantiomer. The column void time (t_0) was estimated using 1,3,5-tri-*tert*-butylbenzene for normal phase and for polar-organic elution while in reversed phase t_0 was considered to be equal to the peak of the solvent front.

RESULTS AND DISCUSSION

Among the most useful and versatile chiral stationary phases (CSPs) are derivatives of polysaccharides.[18,19] These phases have been used extensively, with success, not only in the analytical mode[20] but also in the preparative scale[21] and proved to be effective in normal, reversed-phase and polar mode elution.[22-26] Recent results in the use of polysaccharide-based phases on multimodal elution, in which the performances of the phases were maintained from one elution mode to another, prompted us to attempt a similar strategy.[27-29] Five pairs of xanthonolignoids (**1-5**) were evaluated in carbamate chiral stationary phases of cellulose

(CSP1) and amylose (CSP2, CSP3 and CSP4), all under normal, reversed-phase and polar organic conditions.

A number of publications have described the power of resolution for the chiral phase *tris*-3,5-dimethylphenhylcarbamates of cellulose (CSP1) and its analogue of amylose (CSP2),[16,19,30,31] and their complementary resolving abilities.[31] The *tris*-(S)-1-phenylethylcarbamate (CSP3) and *tris*-3,5-dimethoxyphenylcarbamate (CSP4) also showed some interesting differences in the enantioselectivity.[16,17,30,32] It has been reported that most of the polysaccharide *tris*-phenylcarbamate derivatives form a lyotropic crystalline phase in highly concentrated solution and when coated on silica gel present an ordered structure in which the phenylcarbamate groups are regularly arranged.[33] The enantioselectivity of the derivatized amylose- and cellulose-CSPs is generally attributed to the degree of steric fit of the enantiomers in the "chiral cavity" of the CSP. The carbamate groups on the CSP can interact with solutes, in this case with kielcorins **1-5**, through hydrogen bonding using the C=O and N-H groups, through dipole-dipole interactions using the C=O moiety and through $\pi-\pi$ interactions.[18,31] All the compounds contain similar possibilities of hydrogen bonding or hydrophobic moietys to interact with the CSPs, but attractive interactions can be different due to different accessibility and electronic density. Chiral discrimination can result from differences in their steric fit in the chiral cavities of the CSPs. In fact, the analytes can be grouped according their global shape in: "pistol" (**1**,**2**), "long-chair" (**3**,**4**), and "linear" (**5**) (Fig. 1).

The results on CSP2-4 using the same mobile phase conditions are summarised in Table I-III, respectively. For some individual compounds small modifications on the mobile phase composition were made in order to improve resolution. The cellulose *tris*-3,5-dimethylphenhylcarbamates (CSP1) was not able to resolve any of the 5 analytes in the systematic chromatographic conditions presented ($\alpha<1.4$, $0<Rs<0.5$).

Influence of the mobile phases on retention and enantioseparation

Normal-phase conditions (hexane-alcohol eluents)

Separations of analytes **1-5** were first performed on the four CSPs (CSP1-CSP4) with hexane-ethanol as the mobile phase. When decreasing the polar modifier concentration in normal phase conditions from 30 to 20% of ethanol, enantioselectivity factor (α) and resolution factor (Rs) for the pairs of enantiomers **1-5** were not significantly affected. Thus, for example, separation factors for *rac-trans*-kielcorin E (**5**) on CSP3, slightly increased from

α=1.3, Rs=1.1 to α=1.4, Rs=1.2 when changing from 30 to 20% the content of ethanol, while selectivity and resolution factors for *rac-cis*-kielcorin C (**2**) decreased from α=1.2, Rs=0.8 to α=1.1, Rs<0.5 from hexane-ethanol (70:30) to hexane-ethanol (80:20) (Table II). Decreasing the concentration of ethanol results in significantly increases of the retention factors for all analytes.

Generally, when using amylose derivatives as chiral selectors (CPS2-4) the resolution (Rs) decreased on changing the modifier from ethanol to 2-propanol, whereas when using cellulose based phase (CSP1) selectivity and resolution factors were poor except for *rac-trans*-kielcorin D (**3**) where the selectivity (α=1.3) improved with 2-propanol. In fact, using hexane-2-propanol (90:10) only analyte **2** was partially resolved, on the amylose-based CSP3 (Table II); in contrast best performances could be observed when ethanol was used. Interestingly for *rac-trans*-isokielcorin D (**4**), the enantioseparation factors (α, Rs) on CSP3 decreased as the size of the modifier increased (Table II).

Among the various sets of conditions used, CSP2 was apparently the best choice for kielcorins **1** and **4** and CSP3 for kielcorins **2, 3** and **5**. On the CSP4 and CSP1, the resolution was very poor under normal-phase conditions. Furthermore, the very long retention times are a drawback of this elution method.

Reversed-phase conditions (aqueous-organic eluents)

Generally retention factors increased significantly for the analytes studied with increasing amounts of water. Changing the nature of the organic eluent from methanol to the same amount of acetonitrile decreased k and α.

In reversed-phase conditions, CSP2 provided the best separations for analytes **1, 4** and **5** (Table I). Although partial resolution was obtained with CSP3 for kielcorins **1, 2** and **4**, higher retention factors were observed (Table II). On CSP1 and CSP4 (Table III), poor resolution occurred.

Polar organic conditions

Attention has also been focused on the use of mobile phase with high amounts of organic modifier, the so-called polar-organic mode.[27] Polar organic eluents may offer the advantages of alternative chiral recognition mechanisms and higher solubility of some analytes. Additionally they are easily removed from the analytes which may appear especially useful in preparative scale applications[34] and simulated moving bed chromatography.[35] Poor resolutions and large retention factors of analytes **1-5** in normal and reversed phase conditions

prompt us to analyse kielcorins **1-5** in polar organic conditions, including single polar organic eluents (2-propanol, ethanol, methanol, acetonitrile).

High enantioselectivities and resolution factors were observed for analytes **1**, **4** and **5** in methanol on CSP2 (Table I) whereas 2-propanol and ethanol appeared to be more suitable for analytes **2** and **3**, respectively. Thus, for example, enantiomers of analyte **4** were better resolved with methanol than with ethanol and 2-propanol (Rs= 2.40, 1.70 and 1.60, respectively) on CSP2. This kind of behaviour was not observed for CSP4. On this column, analyte **3** was best-resolved using methanol; the performance for analytes **4** and **5** improved with ethanol (Table III). The analyte **4** on CSP4, was slightly resolved with ethanol but no enantioseparation at all was observed with methanol (Table III). When acetonitrile was used no enantioseparation was observed for all kielcorins (**1-5**).

The amylose 3,5-dimethylphenylcarbamate (CSP2) exhibits the best performance for analytes **1-2** and **4-5**. Simply using single polar organic eluents, a baseline enantioseparation was observed for three analytes (**1**, **4** and **5**) and partially resolution for the other two (**2** and **3**) in this CSP (Table I). Some specificity on CSP4 was observed for enantiomers separation of analytes **3** and **5**. Kielcorins **1-5** exhibited very low k values (0.23-0.90) on CSP3 and partial resolution was only observed for analytes **1** and **4** (Table II). No enantioresolution was observed on CSP1 for analytes **1-5**, similarly to normal and reversed-phase conditions.

Subsequently, mixtures of polar organic modifier were used as mobile phases in order to improve the separation performance. Generally, ethanol-methanol mixtures gave higher enantioselectivity and resolution for analytes **1-4** and methanol-acetonitrile mixtures were better for analyte **5**. Changing the concentration of methanol in ethanol-methanol mixtures had little effect on retention and resolution in all CSPs for all analytes, except on CSP2 where the variation of the content of methanol was noteworthy: for analyte **3**, retention factors slightly increased with the increase of methanol; using a mixture of EtOH-MeOH (50:50) selectivity and resolution increased significantly (α=2.0, Rs=2.6) when compared to EtOH-MeOH (90:10, α=1.2, Rs=0.7) and to EtOH-MeOH (5:95, α=1.0).

The amylose 3,5-dimethylcarbamate (CSP2) appeared to be the most suitable CSP since three of the five analytes (**3**,**4**,**5**) were resolved with excellent degree of resolution and two analytes (**1**,**2**) were partially resolved (Table I). Polar-organic conditions were found to be the most efficient mobile phase for this class of compounds because good resolution values and short retention times were obtained.

The increased retention times of solutes **1-5** result from corresponding decrease in the molar concentration of the organic modifier in normal or reversed-phase conditions, as would

be normally expected, with the polarity of the mobile-phase modifier controlling the k values of the solutes for all CSPs.[36,37] Under polar-organic conditions decreasing the concentration of methanol in acetonitrile and under reversed-phase conditions changing from methanol to acetonitrile, retention times decreased probably due to the higher polarity of acetonitrile that should lead to fewer interactions by hydrogen bonding between the CSP and the analytes.[37,38]

Under normal phase conditions decreasing the alcohol concentration or under reversed phase conditions increasing the amount of water did not improve significantly the separation factors. This indicates that chiral recognition process was unchanged over the explored alcohol/water concentration range.[36,38] Generally, the same behaviour was observed using ethanol-methanol eluents on all CSPs. However, for compounds having a "long-chair" shape (kielcorins **3** and **4**) increased retentions and separations were observed on CSP2 (Table I). The main reason in these cases could be explained by a changed geometry and/or size of the chiral groove caused by the kind of the alcoholic modifier.

When changing the nature of the organic modifiers it was difficult to find any clear relationship with separation factors. In normal-phase conditions, when changing from hexane-2-propanol to hexane-ethanol, separations for analytes **1-5** changed dramatically on all CSPs. In cellulose derivatized CSPs (CSP1), the most important docking sites for the mechanism of chiral discrimination are considered to be the polar carbamate residues which can interact through hydrogen bonding with complementary groups on the analyte molecule.[39,40] Solvents such as alcohols, compete more effectively for the chiral sites than the analyte.[36] The enantiomeric resolution increased as the size of the alcohol increased, with 2-propanol giving better results. This might be due to the decrease in the capacity of higher alcohols to compete for hydrogen bonding sites because of steric hindrance in cellulose derivatized CSPs.[36,41] On the other hand, in amylose derivatized CSPs (CSP2, CSP3) best results were obtained with lower alcohols (methanol and ethanol). This suggests that hydrogen bonding interactions are probably not predominant. Separations might predominantly involve π–π interactions between aromatic moieties of the analyte and of the stationary phases.[37] In opposite to the hydrogen bonding, π- π interactions might be less favourable in higher alcohols.[26] This may be the explanation for decreasing enantioseparation factors for analyte **4** with hexane-alcohol and single organic eluents (methanol>ethanol>2-propanol).

Influence of the structure of the CSPs on chiral recognition ability

The amylose *tris*-3,5-methylphenylcarbamate phase (CSP2) provided the enantioseparation of all the investigated kielcorins (**1-5**), with good retention factors in polar-

organic conditions, being the first choice for developing a preparative method. The amylose *tris*-(S)-1-phenylethylcarbamate phase (CSP3) was able to enantioresolve four (**1**,**2**,**4**,**5**) out of the five analytes investigated having a complementary ability with the amylose *tris*-3,5-dimethoxyphenylcarbamate phase (CSP4) which only enantioresolve two analytes (**3**,**5**). The cellulose *tris*-3,5-methylphenylcarbamate phase (CSP1) was not appropriate for this type of analytes. The chromatograms shown in Fig. 3 illustrate the best separations for analytes **1-5** using CSP2 and the potential of polar organic conditions as a powerful tool for HPLC enantioseparation.

Considering the CSPs (Fig. 2), CSP2 (amylose *tris*-3,5-dimethylphenylcarbamate) exhibited higher enantiomer resolving ability compared to its cellulosic analogue CSP1. Okamoto's group[18,31] explained the difference between the cellulose and the amylose derivatives by the conformation of the helix of the polysaccharide derivatives: left-handed threefold (3/2) helix for cellulose and left-handed fourfold (4/1) helix for amylose. The enantioselectivity factors obtained on CSP1 and CSP2 were greatly influenced by the alcohol modifier. The enantiomeric pairs of the five analytes (**1-5**) were not resolved on CSP1 when hexane-ethanol was employed. On the other hand, the use of hexane-ethanol on CSP2 (Table I), gave rise to a successful and rapid separation of enantiomers. Again this suggests that π–π interactions between an aromatic moieties of the kielcorin derivatives and the aromatic moieties of the stationary phase can be predominant over hydrogen bonding interactions.

In CSP3 (amylose *tris*-(S)-1-phenylethylcarbamate) some interesting differences in selectivity from the amylose *tris*-3,5-dimethylphenylcarbamate CSP2 were observed. The phenyl ring on CSP2 (Fig. 2) is closer to the amylose ring by one carbon, therefore enhancing the bulkiness of the environment around the amylose ring. Furthermore, the CSP3 contains an additional stereogenic center in the phenylcarbamate unit. This could lead to different structures resulting in distinct chiral recognition ability. The retention factors for analytes **1** and **3-5** were the lowest on the CSP3 under normal phase and polar organic conditions. The opposite was observed under reversed phase conditions, with CSP3 providing the highest retention factors for all kielcorins, except for analyte **2**. This could probably be due to a difference in the size and the geometry of the chiral cavities of CSP3 which could provide a better "fit" for analyte **2** in the chiral cavities, when compared with CSP2. Instead, CSP2 seems to be more adequate for the other analytes, especially **3-5**. This may be ascribed regarding the k values of the structurally related kielcorin shapes on CSP3: "long-chair" (**3-4**)<"linear" (**5**)<"pistol"(**1-2**). The nature of the organic modifiers also seems to influence

both CSPs in a similar manner suggesting that nature of interactions presented in chiral recognitions should be similar on CSP2 and CSP3 for this class of compounds.

The nature of substituents on the aromatic groups influences the polarity of carbamate residues, which consequently must change their resolving ability. In CSP2, a methyl group in the phenyl moiety increases the electron density at the carbonyl oxygen atom of the carbamate allowing alcohols to be strongly adsorbed on the stationary phases through hydrogen-bonding interactions. In CSP4, a methoxy group may interact with analytes containing hydroxyl groups. Since this methoxy is far away from the chiral glucose residue[18,31] this results in lowering resolving ability. In addition, changing from a methyl (CSP2) to a methoxy substituent (CSP4) the change in electronic density on the aromatic moiety of the carbamate group could lead to the decreasing of π–π interactions contributions on CSP4.[42] Balanced enantioseparations could be achieved with these two CSP concerning the nature of the mobile phases and the structural features of the analytes. Thus, on CSP2 enantiomers of analytes **4-5** were resolved with methanol but not with ethanol and the opposite was observed with enantiomers of analyte **3**. Contrary to CSP2, the retention and resolution factors of analytes **4-5** on CSP4 increased from methanol to ethanol and the opposite was observed for analyte **3**. For analytes **4-5** the chiral recognition on CSP4 might be due to a decrease of π–π interactions probably associated to an increase of hydrogen bonding formation. For analyte **3**, hydrogen-bonding contributions should be more important in chiral recognition on CSP2 than on CSP4.

These findings are in accordance with previous results[26,35] and indicate that the intermolecular forces involved in analyte retention and enantioseparation are multivariate. Also for kielcorin derivatives, the behaviour of the separation system depends not only on the mobile phase and stationary phase but also on the steric/electronic nature of the analytes.

CONCLUSION

The enantioseparation of kielcorins was performed by HPLC. For this application, the choice of the suitable CSP proved to be a critical factor. Different types of carbamate chiral stationary phases afforded information about the binding properties of the analytes to the CSPs. The amylose phases demonstrated higher power of resolution than cellulose phase. Results suggested that the role of π-π interactions between the CSPs and the chiral kielcorins are of prevailing importance for enantioseparation. CSP3 and CSP4 showed different chiral

recognition abilities for some enantiomeric pairs compared to CSP2. The analytical results allowed the complete separation of analytes **1-5** on CSP2 or by the combination CSP3-CSP4. Multimodal elution proved to be important to the success of the developed method.

The overall results of this study indicate that the mechanism of chiral recognition for these compounds on phenylcarbamate derivatives of polysaccharides are complex and could be better elucidate by other complementary techniques in future work.

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TABLES AND ILLUSTRATIONS

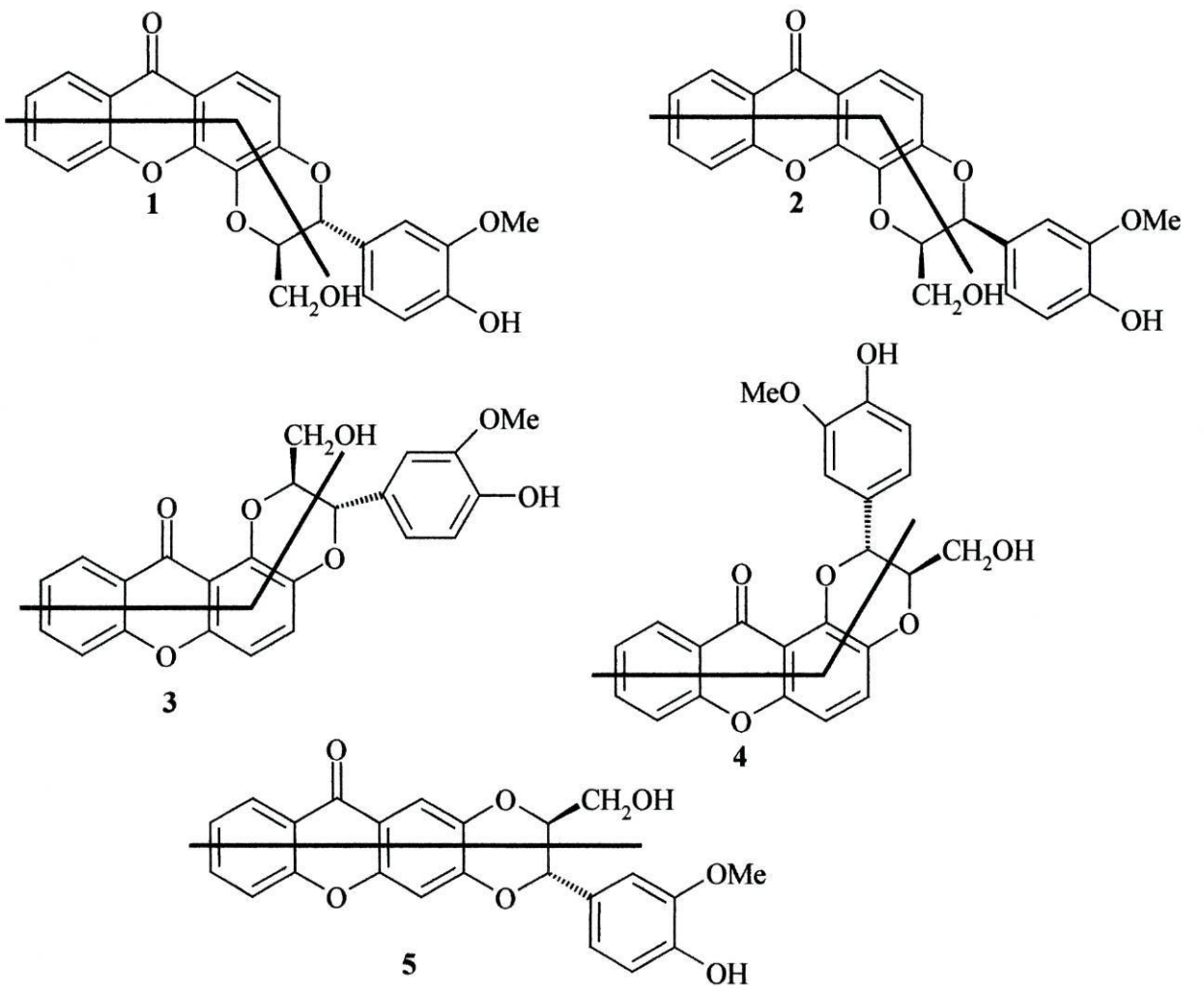


Fig. 1. Global shapes of the investigated analytes: "pistol" for *rac-trans*-kielcorin C (**1**) and *rac-cis*-kielcorin C (**2**), "long-chair" for *rac-trans*-kielcorin D (**3**) and *rac-trans*-isokielcorin D (**4**), and "linear" for *rac-trans*-kielcorin E (**5**).

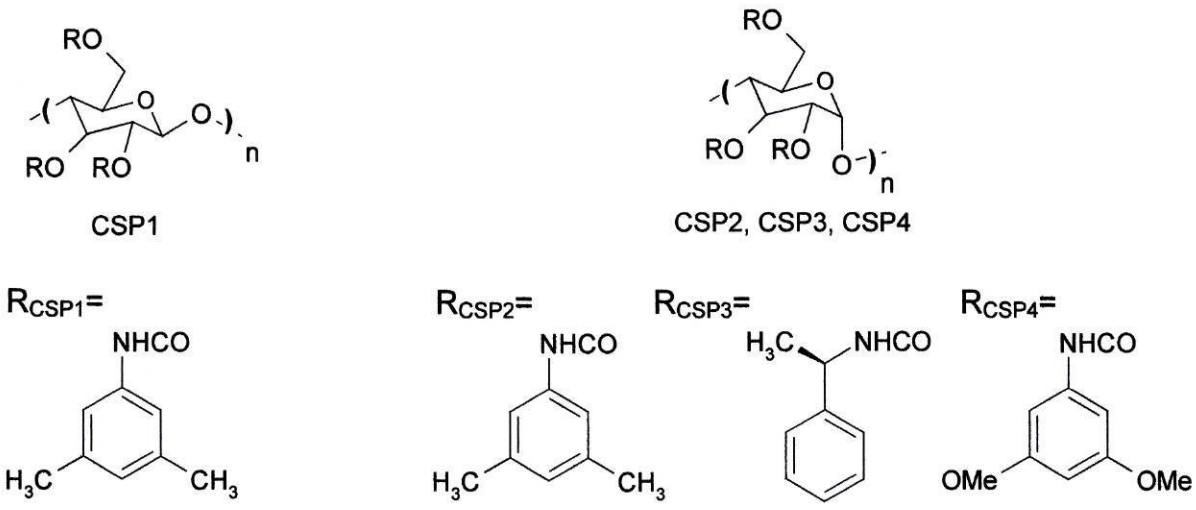


Fig. 2. Structure of chiral stationary phases CSP1-CSP4.

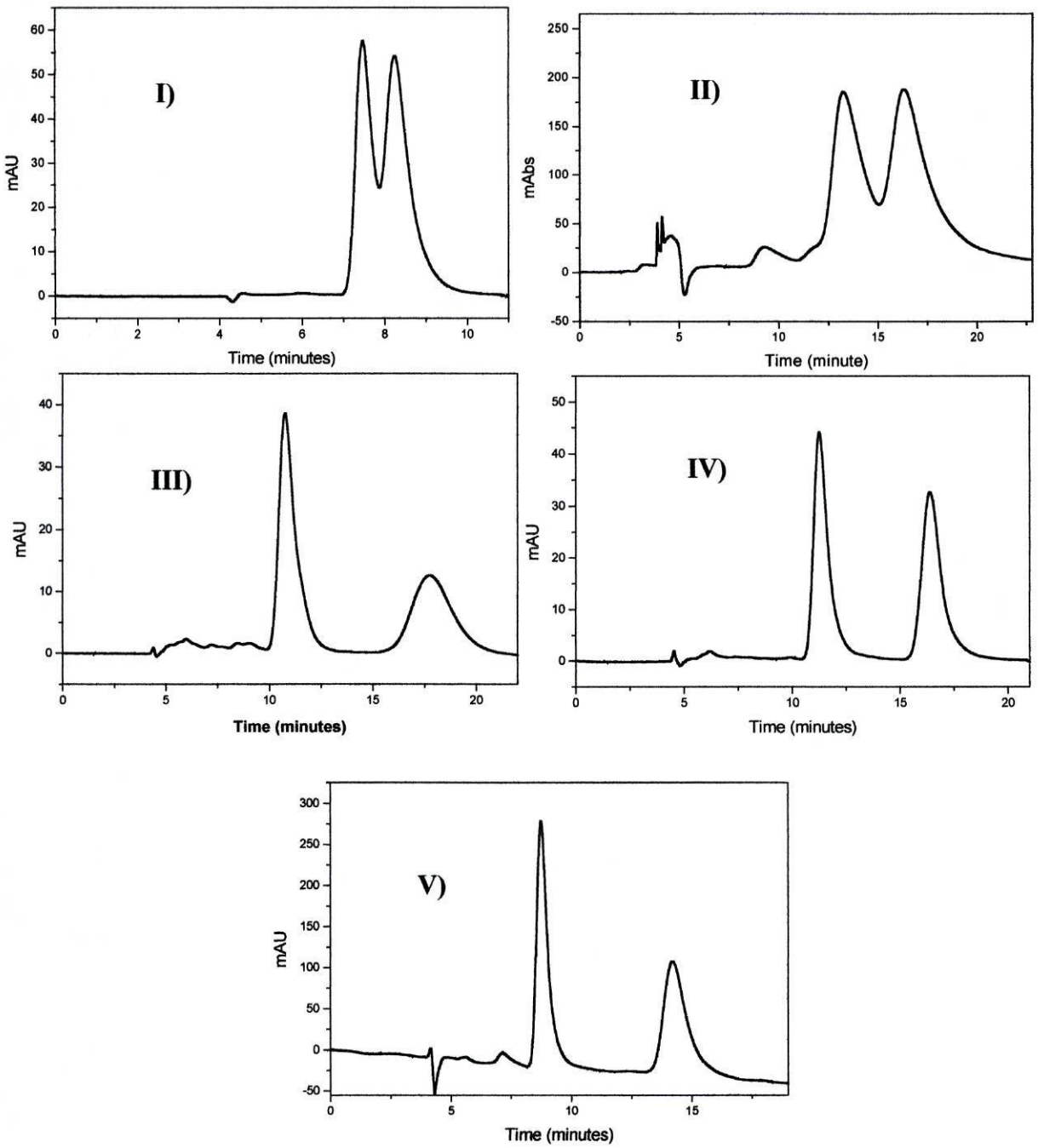


Fig. 3. Chromatograms of the investigated analytes on CSP2 at 0.4 ml.min^{-1}; λ 239nm; using as mobile phases: **I)** 2-propanol-ethanol (3:7) for *rac-trans*-kielcorin C (**1**), **II)** 2-propanol (100%) for *rac-cis*-kielcorin C (**2**), **III)** ethanol-methanol (5:5) for *rac-trans*-kielcorin D (**3**), **IV)** ethanol-methanol (5:5) for *rac-trans*-isokielcorin D (**4**), and **V)** methanol-acetonitrile (5:5) for *rac-trans*-kielcorin E (**5**).

TABLE I. Separation performance [a] of the investigated kielcorin derivatives (1-5) on amylose *tris*-3,5-dimethylphenylcarbamate (CSP2)

Mobile Phase [b]	1			2			3			4			5		
	k_1	α	R_s	k_1	α	R_s	k_1	α	R_s	k_1	α	R_s	k_1	α	R_s
Hex-EtOH (7:3)	6.6	1.2	0.9	5.0	1.3	0.7	6.4	1	nr	3.8	1.3	1.2	>16	-	-
MeOH-TFA 0.1% in H_2O (9:1)	0.8	1.3	0.8	1.0	~1	nc	1.9	1	nr	1.8	1.5	2.3	3.8	2.0	3.1
MeOH-TFA 0.1% in H_2O (8:2)	1.2	1.2	0.8	1.2	~1	nc	3.8	1	nr	3.5	1.4	2.1	>16	-	-
2-prOH	1.5	1.1	0.84	2.3	1.3	0.9	1.4	1	nr	1.8	1.6	1.6	3.5	1.2	0.7
EtOH	0.7	1.4	0.9	1.1	1	nr	1.2	1.2	0.7	1.4	1.4	1.7	2.8	1	nr
MeOH	0.8	1.3	1.2	1.0	~1	nc	1.7	1	nr	1.8	1.5	2.4	3.8	2.0	2.8
ACN	0.7	1	nr	1.1	1	nr	1.4	1	nr	2.3	1	nr	4.6	1	nr
EtOH-MeOH (9:1)	0.6	1.4	0.7	1.1	1	nr	1.1	1.2	0.7	1.4	1.4	1.9	2.6	1	nr
EtOH-MeOH (5:5)	0.6	1.3	0.6	1.0	1.2	0.5	1.5	2.0	2.6	1.5	1.8	3.4	2.8	1	nr
EtOH-MeOH (5:95)	0.8	1.3	0.8	1.1	1.1	<0.5	1.8	1	nr	1.9	1.6	2.0	4.0	1.9	3.0
ACN-MeOH (5:95)	0.7	1.3	0.8	1.0	1.1	<0.5	1.6	1.1	<0.5	1.8	1.5	2.2	3.5	1	nr
ACN-MeOH (5:5)	0.3	1	nr	0.3	1	nr	0.3	1.6	0.8	0.5	1.3	1.0	1.1	2.2	4.0

[a] k1 = retention factor of the first eluted enantiomer, α = enantioselectivity, Rs = resolution factor

[b] Hex = hexane, EtOH = ethanol, 2-prOH = 2-propanol, MeOH =methanol, TFA = trifluoroacetic acid

nr: not resolved.

nc: resolution was not calculated due to lack of accuracy caused by poor resolution.

TABLE II. Separation performance [a] of the investigated kielcorin derivatives (1-5) on amylose *tris*-(S)-1-phenylethylcarbamate (CSP3)

Mobile Phase [b]	1			2			3			4			5		
	k_1	α	R_s	k_1	α	R_s	k_1	α	R_s	k_1	α	R_s	k_1	α	R_s
Hex-EtOH (98:2)										4.6	1.2	1.0			
Hex-EtOH (8:2)	3.6	1.1	0.6	16.8	1.2	1.2	1.28	1	nr	0.9	1.2	0.8	2.7	1.4	1.2
Hex-EtOH (7:3)	2.1	~1	nc	7.4	1.2	1.0	0.6	1	nr	0.5	1.3	<0.5	1.2	1.3	1.1
Hex-EtOH (6:4)													1.1	1.4	1.0
Hex-2-prOH (90:10)	>16	-	-	11.3	1.1	<0.5	7.5	1	nr	6.0	1	nr	>16	-	-
Hex-MeOH (95:5)	1.1	1	nr	>16	-	-	6.0	1.1	<0.5	2.8	1.2	1.0	>16	-	-
MeOH-TFA 0.1% in H_2O (9:1)	6.7	1.1	<0.5	0.9	1.3	<0.5	2.3	1	nr	5.1	1.2	0.6	0.8	nr	nr
EtOH	0.7	1.2	<0.5	0.9	1	nr	0.4	1	nr	0.2	1	nr	0.6	1	nr
MeOH	0.8	1.4	1.3	0.8	1.2	nc	0.3	1	nr	0.4	1.3	0.7	0.7	1	nr
EtOH-MeOH (9:1)	0.7	1.2	0.6	0.9	1	nr	0.3	1	nr	0.4	1.2	<0.5	0.7	1	nr
EtOH-MeOH (5:5)	0.7	1.3	0.9	0.7	1	nr	0.3	1	nr	0.4	1.2	0.6	0.8	1	nr
EtOH-MeOH (5:95)	0.5	1.4	0.9	0.8	1.2	<0.5	0.6	1	nr	0.5	1.3	0.8	0.7	1	nr
ACN-MeOH (5:95)	0.6	1.3	1.0	0.8	1.2	<0.5	0.3	1	nr	0.5	1.3	0.7	0.8	1	nr
ACN-MeOH (5:5)	0.2	1	nr	0.2	1	nr	0.3	1	nr	0.2	1	nr	0.4	1	nr

[a] k1 = retention factor of the first eluted enantiomer, α = enantioselectivity, Rs = resolution factor

[b] Hex = hexane, EtOH = ethanol, 2-prOH = 2-propanol, MeOH =methanol, TFA = trifluoroacetic acid

nr: not resolved.

nc: resolution was not calculated due to lack of accuracy caused by poor resolution.

TABLE III. Separation performance [a] of the investigated kielcorin derivatives (1-5) on amylose *tris*-3,5-dimethoxyphenylcarbamate (CSP4)

Mobile Phase [b]	1			2			3			4			5		
	k_1	α	R_s	k_1	α	R_s	k_1	α	R_s	k_1	α	R_s	k_1	α	R_s
Hex-EtOH (7:3)	11.4	~1	nc	10.0	1.1	nc	7.0	1.1	nc	4.5	1	nr	>16	-	-
MeOH-TFA 0.1% in H_2O (9:1)	0.4	1	nr	0.4	~1	nc	0.1	1	nr	0.7	1	nr	0.4	1	nr
EtOH	0.8	1	nr	0.8	1	nr	0.8	1	nr	1.0	1.1	nc	2.1	1.3	0.8
MeOH	0.7	1.2	<0.5	0.8	1	nr	0.6	1.3	0.8	1.2	1	nr	2.1	1.2	<0.5
ACN	0.3	1	nr	0.3	1	nr	0.3	1	nr	0.5	1	nr	1.8	1	nr
EtOH-MeOH (9:1)	0.8	1	nr	0.8	1	nr	0.8	1	nr	1.0	1.1	<0.5	2.1	1.3	0.8
EtOH-MeOH (5:5)	0.7	~1	nc	0.8	1	nr	0.6	1.2	0.5	0.9	1.2	<0.5	2.3	1.2	0.7
EtOH-MeOH (5:95)	0.7	1.2	0.5	0.8	1	nr	0.6	1.3	0.8	1.1	1	nr	2.2	1.2	<0.5
EtOH-MeOH (2:98)							0.5	1.3	1.0						
ACN-MeOH (5:95)	0.3	1	nr	0.7	1	nr	0.3	1	nr	0.4	1	nr	0.8	1.3	1.6
ACN-MeOH (5:5)	0.3	1	nr	0.3	1	nr	0.7	1.2	0.7	0.4	1	nr	0.6	1.3	1.2

[a] k1 = retention factor of the first eluted enantiomer, α = enantioselectivity, Rs = resolution factor

[b] Hex = hexane, EtOH = ethanol, 2-prOH = 2-propanol, MeOH =methanol, TFA = trifluoroacetic acid

nt: not resolved.

nc: resolution was not calculated due to lack of accuracy caused by poor resolution.

ANEXO V

Pergamon

Bioorganic & Medicinal Chemistry 10 (2002) 3725–3730

BIOORGANIC & MEDICINAL CHEMISTRY

Xanthones as Inhibitors of Growth of Human Cancer Cell Lines and Their Effects on the Proliferation of Human Lymphocytes In Vitro

Madalena Pedro,[a] Fátima Cerqueira,[a] Maria Emília Sousa,[a,b] Maria São José Nascimento[a] and Madalena Pinto[a,*]

[a]*Centro de Estudos de Química Orgânica, Fitoquímica e Farmacologia da Universidade do Porto, Faculdade de Farmácia, Rua Aníbal Cunha, 164, 4050-047 Porto, Portugal*

[b]*Instituto Superior das Ciências da Saúde-Norte, Rua Central da Gandra, 1317, 4580 Gandra, Portugal*

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Abstract—Twenty-seven oxygenated xanthones have been assessed for their capacity to inhibit in vitro the growth of three human cancer cell lines, MCF-7 (breast cancer), TK-10 (renal cancer) and UACC-62 (melanoma). The effect of these xanthones on the proliferation of human T-lymphocytes was also evaluated. Differences on their potency towards the effect on the growth of the human cancer cell lines as well as on the proliferation of human T-lymphocytes can be ascribed to the nature and positions of the substituents on the xanthonic nucleus.
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Introduction

The xanthone structure is a very interesting scaffold for different groups in different positions leading to a large variety of pharmacological activities reported[1–15] namely inhibitors of growth of a variety of tumor cell lines[6–11] as well as modulators of PKC isoforms.[12] Although the anti-inflammatory activity of this group of compounds is documented,[13,14] little information has been accumulated about its interference with the effectors cells of the specific immunity.[15]

As xanthones from natural origin are relatively limited in type and position of the substituents imposed by the biosynthetic pathways, the syntheses of new compounds can afford to enlarge the possibilities of having different nature and positions of the substituents on the xanthonic nucleus that will allow us to rationalize and characterize the structure features that are important to their activity.

Based on these considerations we have synthesized a series of mono-, di- and trioxygenated simple xanthones and investigated their potential antitumor and immunomodulatory activity.

Consequently, this work reports the synthesis and the study of the in vitro effect of twenty seven xanthone derivatives on the growth of three human cancer cell lines, MCF-7 (breast cancer), TK-10 (renal cancer) and UACC-62 (melanoma) and on the mitogenic response of human lymphocytes to phytohemaglutinin (PHA). The aspect of structure–activity relationship within this class of compounds is also tentatively discussed.

Results and Discussion

Chemistry

Chemically xanthones (9*H*-xanthen-9-ones) are heterocyclic compounds with the dibenzo-γ-pyrone framework (Fig. 1). The xanthone nucleus is numbered according to a biosynthetic convention with carbons 1–4 being assigned to acetate-derived ring A and carbons 5–8 to the shikimate-derived ring B. The other carbons are indicated as 4a, 4b, 8a, 9 and 9a for structure elucidation purposes.

*Corresponding author. Tel.: +351-222-057358; fax: +351-222-057358; e-mail: madalena@ff.up.pt

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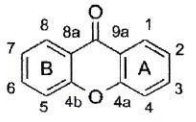


Figure 1. Nucleus of xanthone.

The standard methods for the synthesis of xanthones include the via benzophenone (**I**) and the via diaryl ether (**II**) intermediates (Scheme 1). In the Grover, Shah and Shah classic method the xanthones are obtained by condensation between an *ortho*-oxygenated benzoic acid and an activated phenol, in phosphorous oxychloride and zinc chloride (**a**).[16] The intermediate benzophenone derivatives (**I**) are also accessible through condensation by Friedel–Crafts acylation of appropriately substituted benzoyl chlorides with phenolic derivatives (**b**).[17] While the benzophenone method involves the cyclization of 2,2′-dioxygenated benzophenones (**I**) through a dehydrative or oxidative process (**c**),[17] the diaryl method (Ullman synthesis)[18] was carried out via suitable biphenyl ether intermediate (**II**) with the ring formation by one-step conversion by lithium diisopropylamide (**d**)[19] or by acetyl chloride (**e**).[20]

The compounds **2**, **6** and **24** were synthesized by via (**a**), while the compounds **3**, **4**, **7**, **8**, **13**, **14**, **17**, **18**, **20**–**23**, **25**–**27** were obtained by via (**b**). The diaryl process has led to the synthesis of the compounds **10** and **15** by via (**d**) and the compounds **5**, **9**, **12** and **16** by via (**e**), respectively (see Experimental protocols).

For compounds **10**, **12**, **13**, and **16** are indicated, for the first time, spectral data from INEPT, HETCOR, COSY or HMBC experiments in order to clarify the structural elucidation of these compounds.

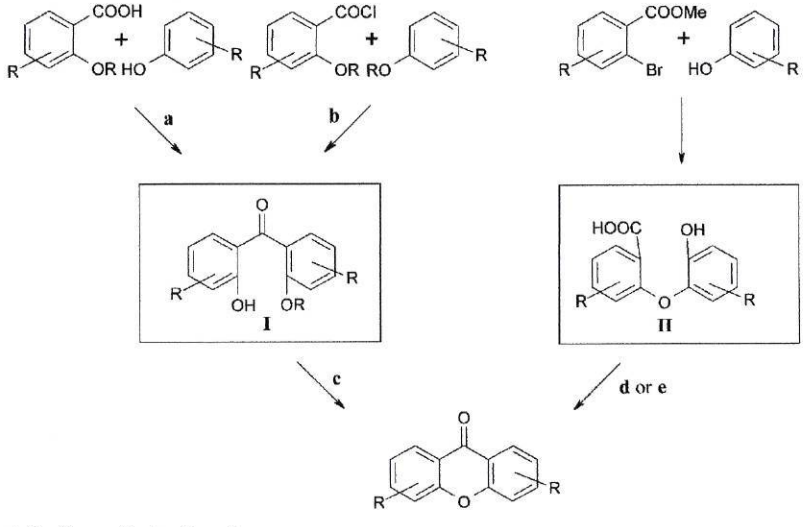
Effect on the growth of human cancer cell lines

The effects of the 27 xanthones on the growth of three human cancer cell lines, MCF-7, TK-10, UACC-62, given in concentrations that were able to cause 50% of cell growth inhibition (GI_{50}), are summarized in Table 1. The growth inhibition effects presented by these xanthones cannot be attributed to a toxic effect, as inferred from the sulforhodamine B (SRB) assay (data not shown).

No capacity to inhibit the growth of the human cancer cell lines was observed with xanthone (**1**) even when tested at concentrations higher than 200 μM, but the introduction of oxygenated groups in the xanthonic nucleus led, in general, to the appearance of a dose dependent growth inhibitory effect. Although this effect showed to be not very strong ($GI_{50} > 50$ μM) for the majority of the xanthone derivatives, some xanthones exhibited interesting growth inhibitory effects on the human cancer cell lines tested with GI_{50} values ≤ 20 μM. Although there was, in general, no discernible cell-type selectivity for the xanthones studied, it was found that 1,2-dihydroxyxanthone (**10**), 3,4-dihydroxyxanthone (**13**), and 2,3-dihydroxy-4-methoxyxanthone (**25**) were significantly ($p < 0.01$) much more active to the melanoma UACC-62 cell line than to MCF-7 and TK-10.

In the mono-oxygenated xanthones with substituents on C(2) or C(4) it is possible to observe drastic changes in the potency of the growth inhibitory activity: substituting OH by OCH_3 on C(2) leads to the appearance of activity while the opposite phenomenon occurs with the same change on C(4).

The nature of the substituents also seems to influence the growth inhibitory effect of the dioxygenated xanthones. The dihydroxylation at 1,2 (**10**), 2,3 (**12**), 3,4 (**13**), and 3,5 (**14**) positions of the xanthonic nucleus was associated with compounds that showed growth inhibitory effects that were significantly ($p < 0.05$) stronger to all the three cell lines than those presented by 1,2- (**15**), 2,3- (**16**), 3,4- (**17**), and 3,5-dimethoxyxantones (**18**). Similarly, the hydroxy and methoxy substituents at 2,1 (**19**), 4,3 (**22**), and 5,3 (**23**) positions were associated with a



Scheme 1. General methods for the synthesis of xanthones.

Table 1. Effects of xanthones on the growth of human cancer cell lines and proliferation of human lymphocytes

No. Compounds	GI_{50} (μM)[a]			IC_{50} (μM)[a]
	MCF-7[b] (breast cancer)	TK-10[b] (renal cancer)	UACC-62[b] (melanoma)	Human lymphocytes[c]
1 Xanthone	>200	>200	>200	>200
2 1-Hydroxy	>200	69.3±10.9	142.9±20.1	125.7±21.7
3 2-Hydroxy	>200	>200	>200	>200
4 3-Hydroxy	125.5±23.8	125.8±7.9	117.9±9.4	>200
5 4-Hydroxy	80.2±9.2	53.8±5.0	66.0±2.7	120.8±12.1
6 1-Methoxy	94.4±8.2	106.2±10.2	78.1±14.5	180.5±34.8
7 2-Methoxy	87.4±8.5	118.7±14.7	59.0±8.2	142.5±23.2
8 3-Methoxy	>200	94.4±14.7	73.8±12.1	53.5±5.1
9 4-Methoxy	>200	>200	>200	>200
10 1,2-Dihydroxy	38.4±2.7	65.8±5.1	14.0±0.3	73.3±2.2
11 1,7-Dihydroxy	57.1±11.0	60.1±9.6	51.3±9.6	20.2±0.2
12 2,3-Dihydroxy	40.6±1.3	61.4±4.3	31.7±3.7	31.3±1.5
13 3,4-Dihydroxy	40.5±1.5	59.2[a]	21.6±2.6	12.2±1.3
14 3,5-Dihydroxy	146.5±13.1	101.6±9.1	77.5±9.8	180.2±5.1
15 1,2-Dimethoxy	>200	>200	136.7±16.6	>200
16 2,3-Dimethoxy	174.1±13.8	174.1±14.1	125.9±21.1	59.9±18.5
17 3,4-Dimethoxy	>200	130.1±11.3	162.1±22.2	42.1±6.3
18 3,5-Dimethoxy	>200	>200	>200	>200
19 2-Hydroxy-1-methoxy	24.1±3.0	35.2±7.6	39.3±10.6	11.6±0.8
20 3-Hydroxy-4-methoxy	>200	>200	>200	178.5±8.9
21 3-Hydroxy-5-methoxy	>200	49.6±16.8	36.9±20.2	>200
22 4-Hydroxy-3-methoxy	108.8±22.7	72.7±9.2	78.5±11.9	92.8±11.8
23 5-Hydroxy-3-methoxy	66.1±12.6	30.9±2.3	37.7±6.7	111.2±8.3
24 1,3-Dihydroxy-2-methyl	21.9±0.4	34.3±3.8	20.0±0.5	84.4±12.4
25 2,3-Dihydroxy-4-methoxy	37.2[d]	76.6±7.0	19.8±1.6	17.4±2.7
26 1-Formyl-4-hydroxy-3-methoxy	75.3±8.6	73.1±9.0	66.7±8.5	80.7±11.7
27 2-Formyl-3-hydroxy-4-methoxy	82.7 ±14.8	128.1±14.3	59.3±7.5	19.2±3.3

[a]Results are expressed as GI_{50} (concentrations of compounds that cause 50% inhibition of cancer cell growth) or IC_{50} (concentrations that cause 50% inhibition of lymphocytes proliferation) and show means±SEM of 3–6 independent observations performed in duplicate.
[b]Doxorubicin was used as positive control in cancer cell lines growth (GI_{50} MCF-7 = 42.8±8.2 nM; GI_{50} TK-10 = 548.0±60.0 nM; GI_{50} UACC-62 = 94.0±9.4 nM).
[c]Cyclosporin A was used as positive control in lymphocytes proliferation (IC_{50} = 0.34±0.04 μM).
[d]Data based on two independently run duplicate experiments.

significant ($p < 0.05$) increase of activity when compared with dimethoxyderivatives (**15**, **17**, **18**) in the same positions. Interestingly the hydroxy-methoxylation at 3,4 (**20**) caused a complete loss of activity of the compound. The improvement of the activity from 3-hydroxy-4-methoxyxanthone (**20**) to 3,4-dihydroxyxantone (**13**) is also remarkable especially for UACC cell line. When 4-hydroxy-3-methoxyxanthone (**22**) and 3-hydroxy-4-methoxyxanthone (**20**) were compared for their activity, it was observed that the shift of the substituents lead to a complete lost of activity in all the cell lines.

In the case of the trisubstituted xanthones tested, the number of compounds is not sufficient to draw any conclusion. However, it is interesting to point out that 1,3-dihydroxy-2-methylxanthone (**24**) was found to have the best inhibitory activity to all the three cell lines.

Effect on human lymphocytes proliferation

The effects of the 27 xanthones against the proliferation of human lymphocytes to PHA, given in concentrations that were able to cause 50% inhibition of proliferation (IC_{50}), are also shown in Table 1.

Xanthone (**1**) showed to be devoid of activity. But the mono-, di- and tri-substitution of the xanthonic nucleus was shown to be, in general, associated with the appearance of a dose-dependent antiproliferative effect. Although the majority of the xanthone derivatives have exhibited a weak inhibitory effect ($IC_{50} > 50$ μM), some oxygenated xanthones, namely 1,7-dihydroxyxanthone (**11**), 3,4-dihydroxyxanthone (**13**), 2-hydroxy-1-methoxyxanthone (**19**), 2,3-dihydroxy-4-methoxyxanthone (**25**), and 2-formyl-3-hydroxy-4-methoxyxanthone (**27**) were found to possess a pronounced inhibitory activity on the mitogenic response of human lymphocytes to PHA ($IC_{50} \leq 20$ μM). No lymphocytoxicity was observed when the human lymphocytes were exposed to the IC_{50} concentrations of these xanthones (cell viability >70%), which leads to the conclusion that their inhibitory activity was associated with cell proliferation rather than to a toxic effect (data not shown).

Table 1 showed that the mono-oxygenated xanthones exhibited in generally weaker antiproliferative effect on T-lymphocytes than di- and trioxygenated xanthones. However, there are interesting structural features within the mono-oxygenated xanthones on their activity.

The nature of the substituents on C(**2**) and C(**4**) of the xanthonic nucleus showed to be very decisive for the antiproliferative effect of these compounds. Thus, 2-hydroxyxanthone (**3**) and 3-hydroxyxanthone (**4**) which OH groups on C(**2**) and C(**3**) were inactive while the presence of OCH3 group in the same positions causes the appearance of the activity in 2-methoxy (**7**) and 3-methoxyxanthone (**8**). In contrast, the activity was lost when substituting OH group on C(**4**) in 4-hydroxy xanthone (**5**) by OCH3 in 4-methoxyxanthone (**9**).

In the dioxygenated xanthones, the nature and positions of the substituents also played an important role in their activity. Thus 1,2-dihydroxyxanthone (**10**) ($IC_{50}=73.3$ μM) is more than 6-fold less active than 2-hydroxy-1-methoxyxanthone (**19**) ($IC_{50}=11.6$ μM) when OH was replaced by OCH3 on C(1) ($p<0.01$). Interestingly, the complete lost of activity was observed when both OH groups were replaced by OCH3 in 1,2-dimethoxy derivative (**15**). The effects of OH and OCH3 substituents were also observed for activity of the xanthones with these substituents on the positions 3,4. As a result, 3,4-dihydroxyxanthone (**13**) ($IC_{50}=12.2$ μM) was more active than 3,4-dimethoxyxanthone (**17**) ($IC_{50}=42.1$ μM) and even more than 3-hydroxy-4-methoxyxanthone (**20**) ($IC_{50}=178.5$), respectively.

Two of the trisubstituted xanthones 2,3-dihydroxy-4-methoxyxanthone (**25**) and the 2-formyl-3-hydroxy-4-methoxyxanthone (**27**) were found to be the most active. The presence of OH or CHO on C(2) seemed to be responsible for the ten-fold increase of the antiproliferative activity of these compounds when compared to 3-hydroxy-4-methoxyxanthone (**20**).

Conclusion

In summary, from all these results it can be inferred that the effects of oxygenated xanthones on the growth of the human cancer cell lines as well as on the proliferation of human T-lymphocytes can be ascribed to the nature, positions and number of substituents on the xanthonic nucleus.

Concerning the effect on the growth of human cancer cell lines, 1,3-dihydroxy-2-methylxanthone (**24**) was found to have the best inhibitory activity to all the three cell lines, while some selectivity for UACC-62 cell line was found for 1,2-dihydroxy (**10**), 3,4-dihydroxy (**13**), and 2,3-dihydroxy-4-methoxyxanthones (**25**).

The dihydroxylation at all the positions of the xanthone derivatives tested was associated with stronger inhibition to all the three cell lines than those presented by the corresponding dimethoxy analogues.

For the effect on human lymphocytes proliferation the dioxygenated derivatives 2-hydroxy-1-methoxyxanthone (**19**) and 3,4-dihydroxyxanthone (**13**) showed the most interesting results for the inhibitory response.

Based on the described results further modifications are underway to improve the activity of these compounds.

Experimental

Chemistry

Purifications of compounds were performed by column chromatography (CC) using Merck silica gel 60 (0.50–0.20 mm), flash chromatography using Merck silica gel 60 (0.040–0.063 mm) and preparative thin layer chromatography (TLC) using Merck silica gel 60 (GF_{254}). Melting points were obtained in a Köfler microscope and are uncorrected. IR spectra were recorded on a Perkin–Elmer 257 in KBr. ^1H and ^{13}C NMR spectra were taken in $CDCl_3$ or DMSO-d_6 at room temperature, on Bruker AC 200, DRX 300 and DRX 500 instruments. Chemical shifts are expressed in δ (ppm) values relative to tetramethylsilane (TMS) as an internal reference. MS spectra were recorded as EI (electronic impact) mode on a Hitachi Perkin–Elmer. Xanthone was purchased from Sigma Chemical Co., St. Louis, USA.

The following xanthone derivatives were synthesized and purified by the described procedures.

The synthesis of the following xanthones has already been described according to the references cited below.

1-Hydroxyxanthone (**2**) (48%), 2-hydroxyxanthone (**3**) (74%), 3-hydroxyxanthone (**4**) (92%), 4-hydroxyxanthone (**5**) (59%), 1-methoxyxanthone (**6**) (86%), 2-methoxyxanthone (**7**) (66%), 3-methoxyxanthone (**8**) (45%), 4-methoxyxanthone (**9**) (14%), 3,4-dimethoxyxanthone (**17**) (61%), 3,5-dihydroxyxanthone (**14**) (55%), 3,5-dimethoxyxanthone (**18**) (35%), 3-hydroxy-5-methoxyxanthone (**21**) (42%), 3-hydroxy-4-methoxyxanthone (**20**) (51%) and 4-hydroxy-3-methoxyxanthone (**22**) (39%) were obtained according to the described procedure.[4] 1,2-Dimethoxyxanthone (**15**) (74%) was obtained[21] and characterized[21,22] according to the described procedures.

2-Hydroxy-1-methoxyxanthone (19) and 1,7-dihydroxyxanthone (11). These xanthones 2-hydroxy-1-methoxyxanthone (**19**) and 1,7-dihydroxyxanthone (**11**) were isolated from *Calophyllum teysmannii* var. *inophylloide* (Guttiferae)[15] and *Cratoxylum maingayi* (Guttiferae),[23] respectively.

1,2-Dihydroxyxanthone (10). The compound **10** was obtained by demethylation of 1,2-dimethoxyxanthone (**15**) according to the described procedure (48%).[24,25] Mp 163–165 °C, methylene chloride/hexane (lit.,[26] 166–167 °C, ethanol aq); UV (EtOH): 261, 243, 232, 203 (33,300, 28,800, 14,200, 11,000); UV (EtOH + NaOH): 311, 236, 222 (76,000, 23,900, 17,000); UV (EtOH + $AlCl_3$): 305, 283, 266, 204 (33,000, 22,900, 87,00); IR (KBr) $\upsilon_{máx}$: 3467, 1606, 1475, 1365, 1288, 1045, 748 cm^{-1}. ^1H NMR (DMSO-d_6, 300.13 MHz) δ:[27,28] 12.45 (1-O*H*), 9.42 (2-O*H*), 8.16 (1H, dd, $J=7.9$, and 1.6 Hz, H-8), 7.81 (1H, ddd, $J=8.4$, 7.0, and 1.6 Hz, H-6), 7.60 (1H, dd, $J=8.4$, and 0.8 Hz, H-5) 7.46 (1H, ddd, $J=7.9$, 7.0, and 0.8 Hz, H-7), 7.32 (1H, d, $J=9.0$ Hz, H-4), 6.96 (1H, d, $J=9.0$ Hz, H-3); ^{13}C NMR (DMSO-d_6, 75.47 MHz) δ: 182.3 (C-9), 155.9 (C-4b), 148.3 (C-4a), 147.6 (C-1), 140.2 (C-2), 136.4 (C-6), 125.4 (C-8), 124.5 (C-3), 124.2 (C-7), 119.3 (C-8a), 118.0 (C-5), 108.8 (C-9a), 106.2

(C-4); EIMS, m/z (% rel. int.): 230 (1, $[M+2]^+$), 229 (1, $[M+1]^+$), 228 (70, $[M^+]$), 199 (13), 126 (5), 115 (4), 78 (95), 63 (100).

2,3-Dihydroxyxanthone (12). The compound **12** was obtained by demethylation of 2,3-dimethoxyxanthone (**17**) according to the described procedure (91%).[25] Mp >330 °C, acetone/hexane (lit.[25] >350 °C ethyl acetate, lit.[29] 293–295 °C chloroform/methanol); UV, IR and EIMS data accord with lit.[29] The unequivocal assignments of carbon resonances, mainly by using 1D selective INEPT and 2D HETCOR experiments, allowed us to correct some ^1H and ^{13}C resonances of **12**,[29] namely the assignment of the proton resonances of H-6 to 7.78 (1H, ddd, $J=8.4$, 6.8, and 1.7 Hz) and H-5 to 7.58 (1H, d, $J=8.4$ Hz), and the carbon resonances of C-2 (144.1), C-3 (151.3), and C-4a (154.2).

3,4-Dihydroxyxanthone (13).[25] (78%) Mp above 330 °C (lit.,[16] 240–241 °C, ethanol aq; lit.,[29] 238–240 °C, methanol); UV, IR and EIMS data accord with lit.[29] the analysis of the ^1H, ^{13}C, COSY, HETCOR and HMBC spectra of **12** permits correction of the literature[29] assignment of the proton resonances of H-6 and H-5 to 7.81 (1H, ddd, $J=8.6$, 6.9, and 1.7 Hz, H-6), and 7.62 (1H, dd, $J=8.6$, and 0.9 Hz, H-5).

2,3-Dimethoxyxanthone (16). The 2-(3′,4′-dimethoxyphenoxy) benzoic acid obtained by an Ullman reaction[18] underwent an appropriate cyclization to **16** according to the described procedure (92%).[20,30] Mp 170–171 °C, methylene chloride/pentane (lit.,[20] 164–165 °C, acetone; lit.,[29] 157–158 °C, chloroform; lit.,[31] 155–159 °C, methylene chloride/heptane; lit.,[32] 165–167 °C, chloroform/petrol); IR and EIMS data accord with lit.[29] The unequivocal assignments of carbon resonances, mainly by using 1D selective INEPT and 2D HETCOR experiments, allowed us to correct some ^1H and ^{13}C resonances of **16**:[17,29] ^1H NMR (DMSO-d_6, 200.13 MHz) δ: 8.16 (1H, dd, $J=7.9$ and 1.5 Hz, H-8), 7.82 (1H, ddd, $J=8.4$, 7.4 and 1.5 Hz, H-6), 7.60 (1H, d, $J=8.4$ Hz, H-5), 7.49 (1H, s, H-1), 7.44 (1H, ddd, $J=7.9$, 7.4, and 0.9 Hz, H-7), 7.20 (1H, s, H-4), 3.94 (3H, s, 3-OCH_3), 3.87 (3H, s, 2-OCH_3); ^{13}C NMR (DMSO-d_6, 50.03 MHz) δ: 174.8 (C-9), 155.7 (C-4b), 155.6 (C-4a), 152.0 (C-3), 146.7 (C-2), 134.8 (C-6), 125.9 (C-8), 124.3 (C-7), 120.9 (C-8a), 118.0 (C-5), 114.0 (C-9a), 104.7 (C-1), 100.5 (C-4), 56.6 (3-OCH_3), 55.9 (2-OCH_3).

1,3-Dihydroxy-2-methylxanthone (24). The compound **24** was obtained (28%) and characterized according to the described procedures.[33,34]

2,3-Dihydroxy-4-methoxyxanthone (25), 1-formyl-4-hydroxy-3-methoxyxanthone (26), 2-formyl-3-hydroxy-4-methoxyxanthone (27). The compounds **25** (74%), **26** (36%) and **27** (28%) were obtained according to the described procedure.[34]

Pharmacology

Fetal bovine serum and RPMI-1640 medium were obtained from Gibco BRL. Cyclosporin A, *N,N*-dimethylformamide (DMF), (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), dimethylsulfoxide (DMSO), doxorubicin hydrochloride, gentamicin, L-glutamine, Histopaque-1077, phytohemagglutinin (PHA), sodium lauryl sulfate (SDS), and sulforhodamine B (SRB) were purchased from Sigma Chemical Co. National Cancer Institute (Bethesda, MD, USA) kindly provided the tumor cell lines.

Stock solutions of xanthones were prepared in DMSO and stored at −20 °C providing uniform samples for re-tests. These frozen concentrates were then diluted to the desired final concentrations with the appropriate solvents immediately prior the biological assays. Final concentrations of DMSO showed no interference with the biological activities tested.

Cell growth assay. The effects of compounds on the growth of human cancer cell lines were evaluated according to the procedure adopted in the NCI's in vitro anticancer drug screening that uses the SRB assay to assess growth inhibition.[35] This colorimetric assay estimates cell number indirectly by staining cellular protein with the protein-binding dye SRB. Three human tumor cell lines were used, MCF-7 (breast cancer), TK-10 (renal cancer), UACC-62 (melanoma). Cells were routinely maintained as adherent cell cultures in RPMI-1640 medium containing 5% heat-inactivated fetal bovine serum, 2 mM glutamine and 50 μg/mL gentamicin at 37 °C in a humidified air incubator containing 5% CO_2. Each cell line was plated at an apropriate density (1.5×10^5 cells/mL to MCF-7 and TK-10, 1.0×10^5 cells/mL to UACC-62) in 96-well plates and allowed to attach overnight. Cells were then exposed for 48 h to serial concentrations of compounds and to the positive control, doxorubicin. Following this incubation period, the adherent cells were fixed in situ washed and dyed with SRB. The bound stain was solubilized and the absorbance was measured at 492 nm in a microplate reader. Growth inhibition of 50% (GI_{50}) was calculated as described elsewhere.[36] Toxicity of xanthones was inferred from the SRB assay by comparing, after 48 h, the absorbance of the wells with xanthone-treated cells with the absorvance of the wells containing untreated cells that were fixed at time zero (time at which compounds were added). Lower absorbances after 48 h of treatment indicate occurrence of cell death instead of growth arrest.

Lymphocyte assays

The effects of xanthones on the mitogenic response of human lymphocytes to PHA (10 μg/mL) were evaluated using a modified colorimetric MTT assay.[37] This assay was previously described by us.[15] Mononuclear cells were isolated from heparinized peripheral blood of healthy volunteers by Histopaque-1077 density centrifugation. The toxicity of xanthones against human lymphocytes was evaluated by an assay based on the ability of viable cells to reduce the colorless tetrazolium salt MTT to a colored formazan product. Briefly, in flat-bottom 96-well plates, $2–3\times10^6$ mononuclear cells/mL in RPMI-1640 medium containing 10% heat-inactivated

fetal bovine serum, 2 mM glutamine and 50 μg/mL of gentamicin were exposed for 24 h to the various concentrations of each sample. Following this incubation period the MTT solution (1 mg/mL) was added. After incubation for 4 h the MTT formazan products were solubilized with SDS/DMF solution (20% SDS in a 50% solution of DMF, pH 4.7) overnight at 37 °C. Absorbance (OD 550 nm) of the colored solution was measured with a plate reader. Lymphocytoxicity, determined in terms of the percentage of viable cells, was present when the viability of the exposed cells was less than 70% of the non-exposed control cells.

Statistics. Unpaired Student's *t*-tests were used. Differences with p values below 0.01 or 0.05 were considered statistically significant. Whenever $GI_{50} > 200$ μM, values of 200 μM were considered to evaluate the statistical significance of differences.

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ANEXO VI

Pergamon

Bioorganic & Medicinal Chemistry 10 (2002) 3219–3227

BIOORGANIC & MEDICINAL CHEMISTRY

Synthesis and In Vivo Modulatory Activity of Protein Kinase C of Xanthone Derivatives

Lucília Saraiva,[a,b] Paula Fresco,[a] Eugénia Pinto,[b] Emília Sousa,[c] Madalena Pinto[c] and Jorge Gonçalves[a,*]

[a]*Serviço de Farmacologia, Faculdade de Farmácia, Universidade do Porto, rua Aníbal Cunha, 164, 4050-047 Porto, Portugal*
[b]*Serviço de Microbiologia, Faculdade de Farmácia, Universidade do Porto, rua Aníbal Cunha, 164, 4050-047 Porto, Portugal*
[c]*Serviço de Química Orgânica, Faculdade de Farmácia, Universidade do Porto, rua Aníbal Cunha, 164, 4050-047 Porto, Portugal*

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Abstract—The modulatory activity of a series of 20 simple xanthones on isoforms α, βI, δ, η and ζ of protein kinase C (PKC) was evaluated using an in vivo yeast phenotypic assay. Hydroxy and/or methoxyxanthones were synthesised. The majority of these compounds caused an effect compatible with activation of PKC and some showed to be more effective than the standard PKC activator (PMA or arachidonic acid). The xanthones tested differ in their efficacy and potency towards individual PKC isoforms and some showed higher selectivities for PKC-δ, -η or -ζ, suggesting that xanthone derivatives can become valuable research tools to elucidate the physiological roles of these isoforms. © 2002 Elsevier Science Ltd. All rights reserved.

Introduction

Protein kinase C (PKC) is a multifamily of serine-threonine kinases. According to their primary structure and cofactor requirements, PKC isoforms are grouped into at least three families: the classical PKCs (cPKCs), which include the isoforms α, βI, βII and γ; the novel (nPKCs), which include the isoforms δ, ε, θ, and η; and the atypical PKCs (aPKCs), which include the isoforms λ and ζ.[1,2] Important roles in cellular functions such as growth, differentiation, tumor promotion and apoptosis[3,4] have been ascribed to PKC. The knowledge of specific roles attributable to individual PKC isoform has been hampered by the lack of isoform-selective drugs. Similarly, the search for selective drugs has also been prevented by the lack of an in vivo method in which effects of drugs on a single mammalian PKC isoform can be tested. The in vitro assays use single isoforms, which are, nevertheless, difficult to isolate and purify. Furthermore, in this type of assays it is not possible to fully reproduce the interactions occurring in vivo between PKC and cellular constituents. These methodological difficulties can be circumvented by using the yeast phenotypic assay. This is an in vivo assay, based on the growth inhibition (reflecting an increase in the cell doubling time) of yeast expressing an individual mammalian PKC isoform, which is proportional to the degree of PKC activation.[5,6]

Natural and synthetic xanthones have been reported to mediate several important biological activities namely anti-tumor,[7–11] anti-inflammatory,[12] anti-thrombotic[13] and neuropharmacological effects.[14–16] Recently, some studies have suggested that these compounds may act, at least in part, by interacting with PKC. Prenylated xanthones have been shown to interact with eukaryote kinases in vitro, namely PKC.[17,18] Norathyriol (1,3,6,7-tetrahydroxyxanthone) reduced the PMA-induced respiratory burst and aggregation, an effect ascribed to a PKC inhibition.[19] Furthermore, 1,7-dihydroxyxanthone can mimick the induction of neuroblastoma cell differentiation caused by PKC activators, such as PMA, an effect blocked by PKC inhibitors.[20]

In the present study, the effects of a series of twenty simple xanthones (9*H*-xanthen-9-ones) on PKC isoforms α and βI (cPKCs), δ and η (nPKCs) and ζ (aPKCs) were characterized using the yeast phenotypic assay. With the exception of the xanthone itself, all xanthones were obtained by synthesis by our group. The criteria for the design of the compounds was the molecular rigidity associated with the xanthone nucleus

*Corresponding author. Tel.: +351-22-2078-932; fax: +351-22-2078-969; e-mail: jorge.goncalves@ff.up.pt

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and the absence of groups with the possibility of conformational isomerism. All the compounds are mono or dioxygenated in one or in the two aromatic rings (Table 1).

Our results show that these simple xanthones interact with PKC causing an effect compatible with a PKC activation. This screening also revealed that these compounds differ on their efficacy and potency to activate individual PKC isoforms and some showed effects higher than the standard PKC activators (PMA for cPKCs and nPKCs and arachidonic acid for aPKCs), what can be explored to develop new selective PKC activators.

Results

Expression of mammalian PKC-α, PKC-βI, PKC-δ, PKC-η and PKC-ζ was confirmed by immunoblotting, using protein extracts of yeast cells, transformed with the gene of a single mammalian PKC isoform, grown in the presence of the transcription inducer (2% galactose). Expression of each of these PKC isoforms resulted in a single antigenic band, which co-migrated with the respective recombinant protein. Protein extracts of transformed yeast cells grown in the absence of galactose did not present antigenic bands (Fig. 1).

A wide range of concentrations of PMA (considered as a standard activator for the classical and novel PKC isoforms) was tested in yeast expressing PKC-α, PKC-βI, PKC-δ and PKC-η. Concentrations higher than 10^{-5} M were not possible to test due to its low solubility in the medium. Since atypical PKC isoforms are not activated by phorbol esters,[21] arachidonic acid was used as the standard activator for PKC-ζ.[22]

In the presence of galactose, PMA caused a concentration-dependent inhibition on the growth of yeast expressing individual cPKCs or nPKCs isoforms, with IC_{50} values (nM) of 112±18 (PKC-α), 243±69 (PKC-βI), 574±37 (PKC-δ) and 0.1±0.01 (PKC-η) ($n=20$). In yeast expressing PKC-ζ, arachidonic acid, but not PMA, caused a concentration-dependent inhibition on the growth of yeast expressing this isoform, with an IC_{50} of 205±33 nM ($n=20$).

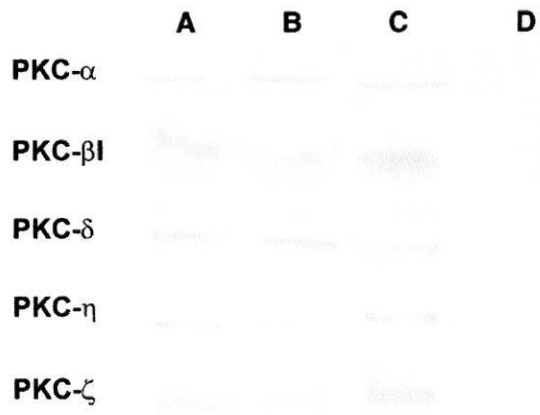
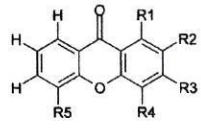


Figure 1. Immunodetection of PKC-α, -βI, -δ, -η and -ζ isoforms expressed in transformed *Saccharomyces cerevisiae* (CG379). Individual immunoblots are presented in a horizontal arrangement and were obtained from proteins extracted from cultures grown in selective medium in the presence of 2% galactose (lanes **A** and **B**; duplicate samples) or in the absence of 2% galactose (lane **D**). Positive controls (lane **C**) were obtained using recombinant proteins PKC-α (MW 76,799 Da), PKC-βI (MW 76,790 Da), PKC-δ (MW 77,517 Da), PKC-η (MW 77,600 Da) and PKC-ζ (MW 67,740 Da).

Table 1. Chemical structures of xanthone derivatives



Compound		R1	R2	R3	R4	R5
1	Xanthone	H	H	H	H	H
2	1-Hydroxyxanthone	OH	H	H	H	H
3	1-Methoxyxanthone	OCH_3	H	H	H	H
4	2-Hydroxyxanthone	H	OH	H	H	H
5	2-Methoxyxanthone	H	OCH_3	H	H	H
6	3-Hydroxyxanthone	H	H	OH	H	H
7	3-Methoxyxanthone	H	H	OCH_3	H	H
8	4-Hydroxyxanthone	H	H	H	OH	H
9	4-Methoxyxanthone	H	H	H	OCH_3	H
10	1,2-Dihydroxyxanthone	OH	OH	H	H	H
11	1,2-Dimethoxyxanthone	OCH_3	OCH_3	H	H	H
12	2,3-Dihydroxyxanthone	H	OH	OH	H	H
13	2,3-Dimethoxyxanthone	H	OCH_3	OCH_3	H	H
14	3,4-Dimethoxyxanthone	H	H	OCH_3	OCH_3	H
15	3,5-Dihydroxyxanthone	H	H	OH	H	OH
16	3,5-Dimethoxyxanthone	H	H	OCH_3	H	OCH_3
17	3-Hydroxy-4-Methoxyxanthone	H	H	OH	OCH_3	H
18	3-Hydroxy-5-Methoxyxanthone	H	H	OH	H	OCH_3
19	4-Hydroxy-3-Methoxyxanthone	H	H	OCH_3	OH	H
20	1,3-Dihydroxy-2-Methylxanthone	OH	CH_3	OH	H	H

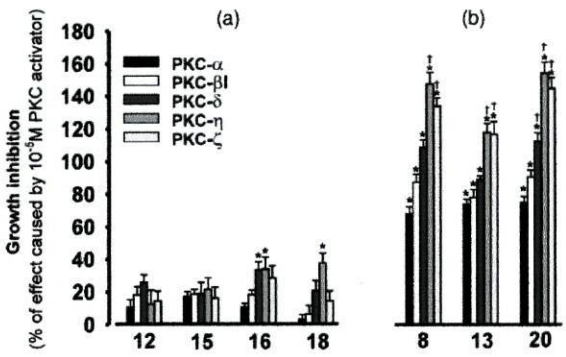


Figure 2. Effects of xanthones on the growth of yeast expressing individual PKC isoforms (α, βI, δ, η or ζ): (a) not effective xanthones; (b), xanthones effective in all PKC isoforms. Yeast cells expressing the indicated mammalian PKC isoform were incubated with xanthones (10^{-5} M) or solvent (DMSO, 0.1% final concentration; see Experimental for details). Shown are means ± SEM of 30–48 determinations. Yeast growth significantly different from solvent: $^{*}p < 0.05$ (paired Student's *t*-test), $^{\dagger}p < 0.05$ significantly higher than the maximal effect caused by 10^{-5} M of PKC activator (unpaired Student's *t*-test).

In the absence of galactose, neither PMA nor arachidonic acid altered yeast growth. Furthermore, the solvent used (DMSO; final concentration 0.1%) did not change yeast growth either in the presence or in the absence of galactose (not shown).

To investigate whether xanthone derivatives (Table 1) could behave as PKC activators, yeast cells expressing PKC-α, PKC-βI, PKC-δ, PKC-η or PKC-ζ were incubated in the presence of each of these compounds. On a first approach each xanthone was tested in a single concentration (10^{-5} M). In the absence of galactose, none of the xanthones tested altered yeast growth (data not shown). In the presence of galactose, the majority of these compounds were able to inhibit the growth of yeast expressing individual PKC isoforms.

The series of 20 xanthone derivatives tested in the present study were first grouped according to their efficacy. Efficacy was evaluated by their ability to elicit a significant growth inhibition, compared to growth in the presence of solvent (DMSO; 0% growth inhibition) and growth in the presence of 10^{-5} M of the respective PKC acti-

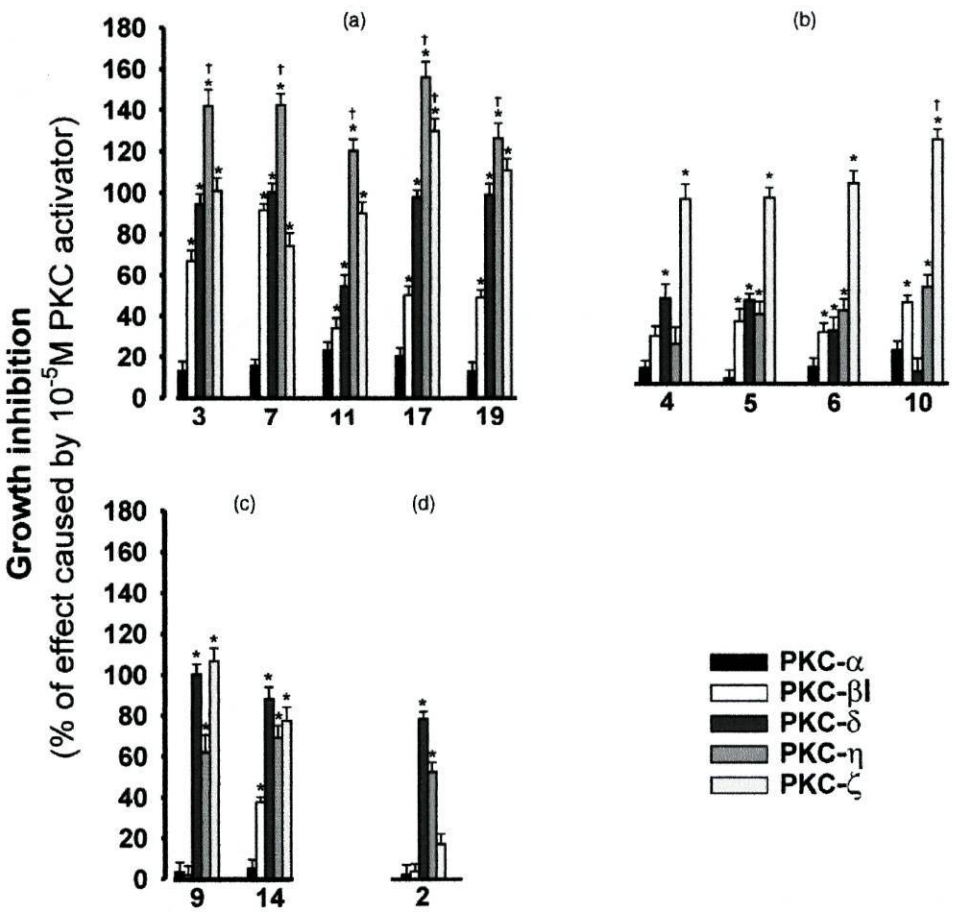


Figure 3. Effects of xanthones showed to be not effective on PKC-α, on the growth of yeast expressing individual PKC isoforms (α, βI, δ, η or ζ): (a) xanthones highly effective on PKC-η; (b) xanthones highly effective on PKC-ζ; (c) xanthones effective on nPKC and aPKC isoforms; (d) xanthones only effective on nPKC isoforms. Yeast cells expressing the indicated mammalian PKC isoform were incubated with xanthones (10^{-5} M) or solvent (DMSO, 0.1% final concentration; see Experimental for details). Shown are means ± SEM of 30–48 determinations. Yeast growth significantly different from solvent: $^{*}p < 0.05$ (paired Student's *t*-test), $^{\dagger}p < 0.05$ significantly higher than the maximal effect caused by 10^{-5} M of PKC activator (unpaired Student's *t*-test).

vator (PMA for PKC-α, -βI, -δ and -η; arachidonic acid for PKC-ζ; 100% growth inhibition). From the series of xanthones tested, a group of four (**12**, **15**, **16** and **18**) was considered not effective in any PKC isoform (Fig. 2a) because they did not cause a growth inhibition that reached at least half of the growth inhibition caused by the respective PKC activator.

A group of three xanthones (**8**, **13** and **20**) was effective in all PKC isoforms (Fig. 2b). It is important to highlight the efficacy of these xanthones on yeast expressing nPKCs (δ and η) and aPKC (PKC-ζ) isoforms. With the exception of xanthone **13** on yeast expressing PKC-δ, all caused inhibition of yeast growth that exceeded those caused by the standard PKC activator. On cPKCs the efficacy of these xanthones was lower than that caused by the standard PKC activator.

The remaining xanthones were effective in some but not in all PKC isoforms. They were tentatively grouped according to the isoform(s) in which they were not effective. A group of 12 xanthones was found to be inactive on PKC-α (Fig. 3). From this group, compounds **3**, **7**, **11**, **17**, and **19** (Fig. 3a) presented high efficacies on PKC-η, causing maximal inhibitions higher than those caused by PMA. Compounds **4**, **5**, **6** and **10** (Fig. 3b) caused a growth inhibition on yeast expressing PKC-ζ much more pronounced than that observed on yeast expressing the other isoforms. Compounds **9** and **14** were similarly effective on yeast expressing nPKCs and aPKC isoforms (Fig. 3c). Compound **2** was effective only on yeast expressing nPKCs isoforms (Fig. 3d).

Compound **1** presented a high efficacy on PKC-η, causing a maximal inhibition higher than that caused by 10^{-5} M PMA (Fig. 4), but differs from the group of compounds in Figure 3a because it was also effective on yeast expressing PKC-α.

The effective compounds were grouped, afterwards, according to their potency by comparing their IC_{50} values, that is the concentration of the xanthone that caused half of the growth inhibition caused by the standard PKC activator PMA (10^{-5} M; arachidonic acid for PKC-ζ). For this purpose, concentration-response curves were obtained and xanthones tested in the concentration range of 10^{-11}–10^{-5} M (see Fig. 5 for typical concentration–response curves obtained for xanthone **20**).

Compounds **8**, **13** and **20**, although effective in all isoforms tested, presented marked differences on their potency (see Table 2). In general they presented an higher potency on yeast expressing PKC-η. Compound **20** presented also a comparable high potency on yeast expressing PKC-ζ. They have also in common a low potency on yeast expressing cPKC isoforms, with the exception of compound **13** on yeast expressing PKC-βI. According to the IC_{50} values, the rank order of potency was, for compound **8**, PKC-η > -δ = -ζ > -α > -βI; for compound **13**, PKC-η > -ζ = -βI > -δ > -α and for compound **20**, PKC-η = -ζ > -δ > -βI > -α (for definition of the rank order of potency, the signal > was applied only when the IC_{50} of the compound placed at the left was significantly lower than that of the IC_{50} placed at the

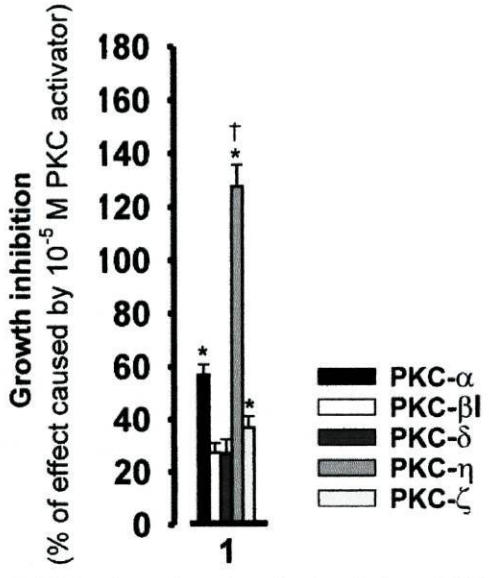


Figure 4. Effects of a xanthone showed to be effective on PKC-α and highly effective on PKC-η, on the growth of yeast expressing individual PKC isoforms (α, βI, δ, η or ζ). Yeast cells expressing the indicated mammalian PKC isoform were incubated with xanthone **1** (10^{-5} M) or solvent (DMSO, 0.1% final concentration; see Experimental for details). Shown are means ± SEM of 24–42 determinations. Yeast growth significantly different from solvent: $^{*}p < 0.05$ (paired Student's *t*-test), $^{\dagger}p < 0.05$ significantly higher than the maximal effect caused by 10^{-5} M of PKC activator (unpaired Student's *t*-test).

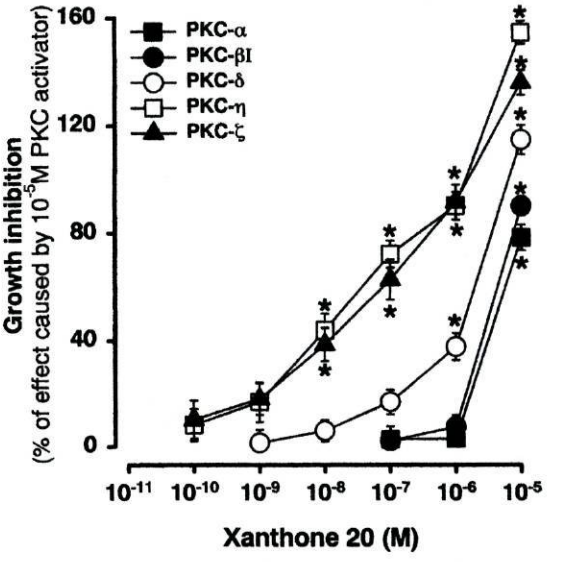


Figure 5. Typical concentration–response curves for the effects of a xanthone (**20**) on the growth of yeast expressing the indicated mammalian PKC isoforms (α, βI, δ, η or ζ). Results are expressed as % of the maximal effect caused by 10^{-5} M of PKC activator (100% growth inhibition was considered in each isoform that caused by 10^{-5} M of PKC activator). Shown are means ± SEM of 20–32 determinations. Yeast growth significantly different from solvent: $^{*}p < 0.05$ (paired Student's *t*-test).

Table 2. IC_{50} values of xanthone derivatives at the mammalian PKC-α, -βI, -δ, -η or -ζ

Compound	IC_{50} (nM)				
	PKC-α	PKC-βI	PKC-δ	PKC-η	PKC-ζ
1	6300±812**	*ND*	*ND*	7±2	*ND*
2	*ND*	*ND*	2510±630	9579±2055*	*ND*
3	*ND*	3981±744**	50±18**	2±0.7	13±2**
4	*ND*	*ND*	*ND*	*ND*	626±73
5	*ND*	*ND*	*ND*	*ND*	188±41
6	*ND*	*ND*	*ND*	*ND*	16±4
7	*ND*	101±12*	19±2	25±8	2000±986*
8	2512±367**	5000±507**	141±14**	9±2	151±21**
9	*ND*	*ND*	1032±190†	8700±916†	6±2
10	*ND*	*ND*	*ND*	*ND*	501±37
11	*ND*	*ND*	*ND*	3±1	2460±690**
13	2500±577**	126±34**	248±27**	1±0.8	79±16**
14	*ND*	*ND*	5±2	2876±1143*	2522±1090*
17	*ND*	9897±3580†	2410±414†	188±62†	4±0.8
19	*ND*	*ND*	1021±232**	2±1	21±10
20	5623±681**,†	3660±352**,†	1585±127**,†	16±4	28±10

IC_{50} values were considered to be the concentration that caused 50% growth inhibition (assuming that 100% growth inhibition was that caused by 10^{-5} M PMA for PKC -α, -βI, -δ and -η or by 10^{-5} M arachidonic acid for PKC-ζ). Xanthones were tested in a concentration range of 10^{-11}–10^{-5} M. Shown are means±SEM of 20–32 determinations. *ND*; non determinable (when the maximal response reached was lower than 50% growth inhibition). Significant differences: relatively to PKC-δ, $^{*}p < 0.05$; relatively to PKC-η, $^{**}p < 0.05$; relatively to PKC-ζ, $^{\dagger}p < 0.05$ (unpaired Student's *t*-test).

right of the signal; otherwise = was applied; $p < 0.05$; unpaired Student's *t*-test).

Compounds **3**, **7**, **11** and **17** were all inactive on yeast expressing PKC-α and presented high potencies on yeast expressing PKC-η. Compound **7** presented also a comparable high potency on yeast expressing PKC-δ whereas compounds **3** and **17** also presented high potencies on yeast expressing PKC-ζ (see Table 2). According to the IC_{50} values, the rank order of potency obtained was, for compound **3**, PKC-η > -ζ > -δ > -βI; for compound **7**, PKC-η = -δ > -βI > -ζ, for compound **11**, PKC-η > -ζ and for compound **17**, PKC-ζ > -η > -δ > -βI.

Compounds **1** and **11** presented a remarkable potency and selectivity towards PKC-η (see Table 2). Compound **1** presented an IC_{50} value 900 times lower than that for the other isoform in which it was also active (PKC-α). Similarly, compound **11** presented an IC_{50} value 820 times lower than that for the other isoform in which it was active (PKC-ζ).

Compounds **4**, **5**, **6** and **10** presented a high selectivity towards PKC-ζ. For these xanthones IC_{50} values were determinable only on PKC-ζ. Nevertheless, they differ on their potency, being compound **6** the most potent followed by **5** and then by compounds **4** and **10** (see Table 2).

Compounds **9**, **14** and **19** have IC_{50} values determinable only on yeast expressing isoforms of the nPKC and aPKC families (see Table 2). Interestingly, each xanthone showed to be particularly potent on a different PKC isoform: **9** on PKC-ζ; **14** on PKC-δ and **19** on PKC-η.

Compound **2** was active only on yeast expressing nPKC isoforms, with a potency higher on yeast expressing PKC-δ than on yeast expressing PKC-η (see Table 2). However, the IC_{50} values were much higher (in the micromolar range) than those presented by xanthones referred as selective to nPKC isoforms.

Discussion

The yeast phenotypic assay, which uses transformed yeast expressing individual mammalian PKC isoforms, has been proposed as an alternative rapid and simple experimental assay for the study of PKC-activating potential of drug candidates. In the present study the mammalian PKC-α, -βI, -δ, -η or -ζ isoforms were expressed in the same yeast strain. After exposure to the PKC activator (PMA or arachidonic acid), a concentration-dependent growth inhibition was detected in yeast expressing each of the PKC isoforms tested. Since the PKC activator-induced growth inhibition was observed only when there was expression of a mammalian PKC isoform (as confirmed by immunoblotting) it is assumed that, under the present experimental conditions, the drug-induced growth inhibition reflects activation of the expressed mammalian PKC isoform.

The majority of the xanthones tested revealed effects compatible with activation of PKC isoforms of the cPKC, nPKC and aPKC families, although, in general, they showed a very low activity on isoforms of the cPKC family, particularly on PKC-α: among the 20 xanthones tested only four (**1**, **8**, **13** and **20**) were able to activate this isoform.

PKC activators like phorbol esters and arachidonic acid, activate PKC by interacting at the regulatory domain[23] but by a distinct mechanism.[24] To activate

PKC, phorbol esters seem to require two cysteine-rich zinc-fingers at the C1 region of the regulatory domain (C1a and C1b).[4] Atypical PKC isoforms lack the second cysteine-rich motif, what explains the inactivity of phorbol esters on isoforms of this family. Arachidonic acid activates PKC by a mechanism that does not require the repeated zinc finger motifs and, thus, arachidonic acid acts as an universal PKC activator.[25] Xanthones activated isoforms of all PKC families, including isoforms of the aPKC family, suggesting that xanthones interact with PKC by a mechanism similar to that of arachidonic acid.

The xanthones tested showed marked activities on nPKC and aPKC. Some were more potent than the standard PKC activator and presented a remarkable selectivity for a specific isoform. These simple xanthones were especially active on PKC-η and on PKC-ζ, in which only four compounds (**4**, **5**, **6** and **10**) and two compounds (**1** and **2**) were inactive, respectively.

Efforts have been made in the past by several groups to study, by using molecular modeling approaches, structure–activity relationships and to establish a common pharmacophore shared by structurally different PKC-activators, despite the lack of 3-D-structural data for individual PKC isoforms.[26] Our study shows that small structural changes on the xanthone framework can lead to dramatic alterations of its activity/selectivity towards individual PKC isoforms. Furthermore, it revealed some insights about the structural requirements of xanthones for PKC activation. Positions 3 and 5 do not seem to be recommendable for introduction of hydroxy/methoxy substituents, once xanthones **15**, **16** and **18** were not effective in any PKC isoform studied. On the other hand, the methoxylation of positions 2 and 3 were shown to be the most favoured substitution to achieve activity in all PKC isoforms tested (compound **13** compared with compound **12**).

The present study revealed new compounds which showed high selectivity for individual PKC isoforms: compound **14** for PKC-δ and compounds **4**, **5**, **6**, **9** and **10** for PKC-ζ, isoforms reported to be of crucial importance in the apoptotic process;[3] and compounds **1** and **11** for PKC-η, an isoform suggested to be involved in growth, differentiation and carcinogenesis of epithelial tissues.[27] Therefore xanthones can be useful for a detailed study of the physiological and pathophysiological roles of PKC isoforms.

Conclusions

This study shows that simple xanthones can interact with mammalian PKC isoforms of the cPKC, nPKC and aPKC families, causing an effect compatible with PKC activation. The high potency and selectivity presented suggests that xanthones can become an important group to look for potent and isoform-selective PKC activators which can be useful to elucidate the physiological and pathophysiological roles of individual PKC isoforms.

Experimental

Chemistry

Purifications of compounds were performed by column chromatography using Merck silica gel 60 (0.50–0.20 mm), flash chromatography using Merck silica gel 60 (0.040–0.063 mm) and preparative thin layer chromatography (tlc), using Merck silica gel 60 (GF_{254}). Melting points were obtained in a Köfler microscope and are uncorrected. IR spectra were recorded on a Perkin-Elmer 257 in KBr. ^1H and ^{13}C NMR spectra were taken in $CDCl_3$ or DMSO-d_6 at room temperature, on Bruker AC 200 and DRX 300 instruments. Chemical shifts are expressed in δ (ppm) values relative to tetramethylsilane (TMS) as an internal reference. MS spectra were recorded as EI (electronic impact) mode on a Hitachi Perkin-Elmer.

Xanthone (**1**) was purchased from Sigma Aldrich (Sintra, Portugal). The following materials were synthesized and purified by the described procedures. The synthesis of the following compounds have already been described:[28] 1-hydroxyxanthone (**2**), 1-methoxyxanthone (**3**), 2-methoxyxanthone (**5**), 2-hydroxyxanthone (**4**), 3-hydroxyxanthone (**6**), 3-methoxyxanthone (**7**), 4-hydroxyxanthone (**8**), 4-methoxyxanthone (**9**), 3,5-dihydroxyxanthone (**15**), 3,5-dimethoxyxanthone (**16**), 3-hydroxy-5-methoxyxanthone (**18**), 3,4-dimethoxyxanthone (**14**), 3-hydroxy-4-methoxyxanthone (**17**) and 4-hydroxy-3-methoxyxanthone (**19**).

The synthesis of the following compounds have been carried out as follows.

1,2-Dihydroxyxanthone (10). Obtained by demethylation of **11** according to the procedure described.[29,30] Mp 163–165 °C, methylene chloride/hexane,[31] 166–167 °C, ethanol aq); $\upsilon_{máx}$ (cm^{-1}) KBr: 3467, 1606, 1475, 1365, 1288, 1045, 748; ^1H NMR (DMSO-d_6, 300.13 MHz) δ: 12.45 (1-O*H*), 9.42 (2-O*H*), 8.16 (1H, dd, J=7.9 and 1.6 Hz, H-8), 7.81 (1H, ddd, J=8.4, 7.0 and 1.6 Hz, H-6), 7.60 (1H, dd, J=8.4 and 0.8 Hz, H-5) 7.46 (1H, ddd, J=7.9, 7.0 and 0.8 Hz, H-7), 7.32 (1H, d, J=9.0 Hz, H-4), 6.96 (1H, d, J=9.0 Hz, H-3); ^{13}C NMR (DMSO-d_6, 75.47 MHz) δ: 182.3 (C-9), 155.9 (C-4b), 148.3 (C-4a), 147.6 (C-1), 140.2 (C-2), 136.4 (C-6), 125.4 (C-8), 124.5 (C-3), 124.2 (C-7), 119.3 (C-8a), 118.0 (C-5), 108.8 (C-9a), 106.2 (C-4); MS m/z (rel int): 230 (1, $[M+2]^{+\cdot}$), 229 (1, $[M+1]^{+\cdot}$), 228 (70, $[M^{+\cdot}]$), 199 (13), 126 (5), 115 (4), 78 (95), 63 (100).

1,2-Dimethoxyxanthone (11).[32] Mp 133–135 °C, methylene chloride/hexane,[31] 130 °C; $\upsilon_{máx}$ (cm^{-1}) KBr: 3488, 3436, 3377, 1662, 1461, 1292, 1085, 1049, 742; ^1H NMR (DMSO-d_6, 200.13 MHz) δ: 8.12 (1H, dd, J=7.9 and 1.7 Hz, H-8), 7.81 (1H, ddd, J=7.6, 7.4 and 1.7 Hz, H-6), 7.63 (1H, d, J=9.3 Hz, H-4), 7.56 (1H, d, J=7.4 Hz, H-5), 7.44 (1H, dd, J=7.9, 7.6 and 1.1 Hz, H-7), 7.40 (1H, d, J=9.3 Hz, H-3), 3.86 (3H, s, 1-OCH_3), 3.80 (3H, s, 2-OCH_3); ^{13}C NMR (DMSO-d_6, 50.03 MHz) δ: 175.2 (C-9), 154.7 (C-4b), 150.3 (C-2), 148.9 (C-1), 147.5 (C-4a), 135.0 (C-6), 126.0 (C-8), 123.9 (C-7), 121.4 (C-8a), 120.7 (C-5), 117.6 (C-3), 116.4 (C-9a), 113.2 (C-4), 61.0

(1-OCH_3), 56.6 (2-OCH_3); MS *m/z* (rel int): 258 (3, $[M+2]^{+\cdot}$), 257 (25, $[M+1]^{+\cdot}$), 256 (100, $[M^{+\cdot}]$), 241 (83), 227 (45), 223 (18), 213 (59), 196 (14), 184 (10), 170 (11), 155 (13), 139 (15), 127 (13), 121 (18), 114 (12), 104 (6), 92 (11), 84 (5), 77 (17), 63 (12).

2,3-Dihydroxyxanthone (12). Obtained by demethylation of **14** according to the procedure previously described.[30] Mp >330 °C, acetone/hexane[30] >350 °C ethyl acetate,[33] 293–295 °C chloroform/methanol); $\upsilon_{máx}$ (cm^{-1}) KBr: 3400, 1624, 1464, 1311, 1290, 1145, 746; ^1H NMR (DMSO-d_6, 300.13 MHz) δ: 8.12 (1H, dd, *J* = 7.5 and 1.6 Hz, H-8), 7.78 (1H, ddd, *J* = 8.4, 6.8 and 1.7 Hz, H-6), 7.58 (1H, d, *J* = 8.4 Hz, H-5), 7.43 (1H, s, H-1), 7.42 (1H, ddd, *J* = 7.5, 6.8 and 1.9 Hz, H-7), 6.92 (1H, s, H-4); ^{13}C NMR (DMSO-d_6, 75.47 MHz) δ: 175.0 (C-9), 155.7 (C-4b), 154.2 (C-4a), 151.3 (C-3), 144.1 (C-2), 134.7 (C-6), 125.9 (C-8), 124.1 (C-7), 120.9 (C-8a), 118.1 (C-5), 113.7 (C-9a), 108.9 (C-1), 103.0 (C-4); MS *m/z* (rel int): 230 (0.34, $[M+2]^{+\cdot}$), 229 (2.4, $[M+1]^{+\cdot}$), 228 (16, $[M^{+\cdot}]$), 200 (0.96), 171 (0.80), 126 (1.8), 115 (0.96), 100 (8), 78 (86), 63 (100).

2,3-Dimethoxyxanthone (13). The 2-(3′,4′-dimethoxyphenoxy)benzoic acid obtained initially by an Ullman reaction[34] underwent an appropriate cyclization to **13** according to the procedure previously described[35,36] Mp 170–171 °C, methylene chloride/pentane[36] 164–165 °C, acetone,[33] 157–158 °C, chloroform); $\upsilon_{máx}$ (cm^{-1}) KBr: 3500, 3440, 3407, 1649, 1466, 1313, 1234, 1005, 762; ^1H NMR (DMSO-d_6, 200.13 MHz) δ: 8.16 (1H, dd, *J* = 7.9 and 1.5 Hz, H-8), 7.82 (1H, ddd, *J* = 8.4, 7.4 and 1.5 Hz, H-6), 7.60 (1H, d, *J* = 8.4 Hz, H-5), 7.49 (1H, s, H-1), 7.44 (1H, ddd, *J* = 7.9, 7.4, and 0.9 Hz, H-7), 7.20 (1H, s, H-4), 3.94 (3H, s, 3-OCH_3), 3.87 (3H, s, 2-OCH_3); ^{13}C NMR (DMSO-d_6, 50.03 MHz) δ: 174.8 (C-9), 155.7 (C-4b), 155.6 (C-4a), 152.0 (C-3), 146.7 (C-2), 134.8 (C-6), 125.9 (C-8), 124.3 (C-7), 120.9 (C-8a), 118.0 (C-5), 114.0 (C-9a), 104.7 (C-1), 100.5 (C-4), 56.6 (3-OCH_3), 55.9 (2-OCH_3); MS *m/z* (rel int): 258 (1, $[M+2]^{+\cdot}$), 257 (8, $[M+1]^{+\cdot}$), 256 (40, $[M^{+\cdot}]$), 241 (11), 185 (3), 170 (3), 142 (3), 78 (89), 63 (100).

1,3-Dihydroxy-2-methylxanthone (20). Obtained according to the procedure previously described.[37,38]

General methods for the in vivo yeast phenotypic assay

Yeast nitrogen base was from DIFCO (Merck Portugal, Lisboa, Portugal). The kit for protein quantification was from Pierce (Biocontec, Lisboa, Portugal). The secondary alkaline phosphatase-conjugated anti-rabbit IgG detection kit (AP-10), recombinant proteins PKC-α (PK11), PKC-βI (PK16), PKC-δ (PK31), PKC-η (PK46) and PKC-ζ (PK41) were from Oxford Biomedical Research (LabClinics, Barcelona, Spain). Nitrocellulose membranes and all the reagents for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and immunoblots were from BioRad (PACI, Lisboa, Portugal). Acid-washed glass beads, antibodies to PKC-α, PKC-βI, PKC-δ, PKC-η and PKC-ζ, aprotinin, arachidonic acid sodium salt, leupeptin, pepstatin A, phenylmethylsulfonyl fluoride, phorbol 12-myristate 13-acetate (PMA) were from Sigma Aldrich (Sintra, Portugal). All other chemicals used were of analytical grade.

Yeast transformation and cell cultures. Constructed yeast expression plasmids YEp52 and YEp51, encoding the cDNA for bovine PKC-α and for rat PKC-βI, respectively (kindly offered by Dr. Heimo Riedel, Wayne University, Detroit, USA) and YEplac181, encoding the cDNA for the rat PKC-δ, mouse PKC-η or PKC-ζ (kindly offered by Dr. Nigel Goode, Royal Veterinary College, London, UK) were amplified in *Escherichia coli* DH5α and confirmed by restriction analysis. The plasmids used contain galactose-inducible transcriptional elements and the leu2 gene for selection. *Saccharomyces cerevisiae* (*S. cerevisiae*; strain CG379; α *ade5 his7–2 leu2–112 trp1–289α ura3–52 Kil-O;* Yeast Genetic Stock Center, University of California, Berkeley, USA) was transformed using the lithium acetate method.[39] To ensure the selection of transformed yeast, cells were grown in leucine-free medium, in 1.5% agar plates, at 30 °C.

For the yeast phenotypic assay, transformed cells were incubated in leucine-free medium, with slow shaking, at 30 °C. The leucine free-medium contained 0.7% yeast nitrogen base, 2% glucose (w/v) or the indicated carbon source, amino acids, purines and pyrimidines, according to the transformed yeast requirements. Galactose (2%; w/v), instead of glucose, was included to induce transcription of the mammalian PKC gene.

Cell lysis and immunoblotting. Cell lysis was performed basically as described.[6] Detergent lysates were separated on 10% SDS-polyacrylamide gels (Mini-Protean II, BioRad, Hercules, CA, USA). Proteins were electrophoretically transferred to nitrocellulose membranes and probed on immunoblots with specific rabbit antibodies to either PKC-α, PKC-βI, PKC-δ, PKC-η or PKC-ζ and with a secondary alkaline phosphatase-conjugated anti-rabbit IgG. Standard recombinants of PKC-α (MW 76,799 Da), PKC-βI (MW 76,790 Da), PKC-δ (MW 77,517 Da), PKC-η (MW 77,600 Da) and PKC-ζ (MW 67,740 Da) were used as positive controls.

Yeast phenotypic assay. Transformed yeast cultures were incubated in leucine-free medium. Optical density values, measured at 620 nm (OD_{620}; Cary 1E Varian spectrophotometer, Palo Alto, CA, USA), were used as an indicator of growth. Transformed yeast were grown to an OD_{620} of approximately 1, collected by centrifugation and diluted to an OD_{620} of 0.05, in medium containing 2% (w/v) galactose (gene transcription inducer) and 3% (v/v) glycerol (alternative carbon source). Diluted cultures (200 μL) were transferred to 96-well microtitre plates and incubated for up to 100 h, with slow shaking at 30 °C, either in the presence of drugs or solvent (DMSO 0.1%; final concentration). Growth was monitored by determining the OD_{620} using a plate reader (BioRad Benchmark Microplate Reader; Hercules, CA, USA). In preliminary experiments, growth curves for individual isoforms were determined

and the duration of the logarithmic and stationary phases identified. Estimation of drug effects was based on OD_{620} measurements at fixed time points (at 65 h for cPKC isoforms or at 48 h incubation for nPKC and aPKC), times occurring during the respective logarithmic phase and when it was reached a 'steady-state growth inhibition' (period of time during which maximal inhibition of growth was reached and remained constant or changed only slightly). In individual experiments, OD_{620} was routinely monitored for up to 100 h to confirm whether the 'fixed time points' chosen were appropriate for all the compounds studied (PMA, arachidonic acid or xanthones). The difference between the maximal OD_{620} reached and that measured at the beginning of incubation was used as an index of yeast growth. Drugs or solvent were added at the beginning and kept throughout the incubation. Yeast growth in the presence of drugs was expressed as percentage of growth observed in parallel experiments in the presence of solvent; it was further transformed into growth inhibition by subtracting that value from 100. Because growth inhibition caused by a maximal concentration of the standard PKC activator varied between isoforms, 100% growth inhibition was assumed to be that caused by 10^{-5} M PMA (or arachidonic acid for PKC-ζ), in order to standardise the maximal inhibition reachable; 0% growth inhibition would occur when growth in the presence of drugs was identical to that in the presence of solvent.

Effects of xanthones were expressed as percentage of that effect. For determination of concentration–response curves, each drug was tested in concentrations ranging from 10^{-11} to 10^{-5} M and the concentration of drug that caused half of the 100% growth inhibition (IC_{50}) was calculated.

Statistical analysis. Results are given as arithmetic means ± SEM and n represents the number of determinations. Differences between means were tested for significance using the paired or unpaired Student's t-test, as indicated in the figure legends. A p value lower than 0.05 was taken to be statistically significant.

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ANEXO VII

Pergamon

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Inhibition of Protein Kinase C by Synthetic Xanthone Derivatives

Lucília Saraiva,[a,b] Paula Fresco,[a] Eugénia Pinto,[b] Emília Sousa,[c] Madalena Pinto[c] and Jorge Gonçalves[a,*]

[a]*Serviço de Farmacologia, CEQOFFUP, Faculdade de Farmácia, Universidade do Porto, rua Aníbal Cunha, 164, 4050-047 Porto, Portugal*
[b]*Serviço de Microbiologia, CEQOFFUP, Faculdade de Farmácia, Universidade do Porto, rua Aníbal Cunha, 164, 4050-047 Porto, Portugal*
[c]*Serviço de Química Orgânica, CEQOFFUP, Faculdade de Farmácia, Universidade do Porto, rua Aníbal Cunha, 164, 4050-047 Porto, Portugal*

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Abstract—The modulatory activity of two xanthones (3,4-dihydroxyxanthone and 1-formyl-4-hydroxy-3-methoxyxanthone) on isoforms α, βI, δ, η and ζ of protein kinase C (PKC) was evaluated using an in vivo yeast phenotypic assay. Both xanthones caused an effect compatible with PKC inhibition, similar to that elicited by known PKC inhibitors (chelerythrine and NPC 15437). PKC inhibition caused by xanthones was confirmed using an in vitro kinase assay. The yeast phenotypic assay revealed that xanthones present differences on their potency towards the distinct PKC isoforms tested. It is concluded that 3,4-dihydroxyxanthone and 1-formyl-4-hydroxy-3-methoxyxanthone may become useful PKC inhibitors and xanthone derivatives can be explored to develop new isoform-selective PKC inhibitors.
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Introduction

Protein kinase C (PKC) is a family of serine-threonine kinases with important roles in cellular functions such as growth, differentiation, tumor promotion and apoptosis.[1] PKC isoforms are grouped into at least three groups: the classical PKCs (cPKCs), which include the isoforms α, βI, βII and γ; the novel PKCs (nPKCs), which include the isoforms δ, ε, θ, and η; and the atypical PKCs (aPKCs), which include the isoforms ζ and λ/ι.[2,3] More recently, a fourth group of structurally distinct PKCs has been identified, the so-called PKC-related kinases (PRKs).[2,3] A new member of the PKC family, PKCμ/PKD has also been reported, but its inclusion remains controversial.[2]

Knowledge of specific roles attributable to individual PKC isoforms has been hampered by the lack of isoform-selective drugs and the search for selective drugs has been difficult because of several methodological limitations. Examples of these limitations are the coexistence of several isoforms in the cells used for in vivo assays, and the difficulties to reproduce in vitro the interactions occurring in vivo between PKC and other cellular constituents. These limitations can be circumvented using the yeast phenotypic assay. This is an in vivo assay, using yeast expressing an individual mammalian PKC isoform, based on the growth inhibition (reflecting an increase in the cell doubling time) which is proportional to the degree of PKC activation.[4,5] In the yeast phenotypic assay, PKC activators cause growth inhibition of transformed yeast[4,5] while PKC inhibitors block the growth inhibition caused by a PKC activator.[6,7]

Natural and synthetic xanthones have been reported to mediate several important biological activities namely anti-tumor,[8–12] anti-inflammatory,[13] anti-thrombotic[14] and neuropharmacological effects.[15–17] Recently, it has been suggested that these compounds may act, at least in part, by interacting with PKC, and cause effects compatible not only with PKC activation[18] but also with PKC inhibition. Prenylated xanthones were shown to be potent inhibitors of eukaryote kinases in vitro, including PKC[19] and norathyriol (1,3,6,7-tetrahydroxyxanthone) reduced the PMA-induced respiratory burst and aggregation, an effect ascribed to inhibition of PKC.[20]

*Corresponding author. Tel.: +351-22-2078932; fax: +351-22-2078969; e-mail: jorge.goncalves@ff.up.pt

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The modulatory activity of a series of twenty-two simple xanthones (9*H*-xanthen-9-ones) on PKC isoforms α and βI (cPKCs), δ and η (nPKCs) and ζ (aPKCs) were characterized in our laboratory using the yeast phenotypic assay. The majority of these compounds caused an effect compatible with PKC activation.[21] In the present study, we report that two of the xanthones tested (3,4-dihydroxyxanthone and 1-formyl-4-hydroxy-3-methoxyxanthone) caused effects compatible with PKC inhibition. The effects of these xanthones were compared with two established potent and selective PKC inhibitors (chelerythrine[22] and NPC 15437[23,24]). The two compounds presented differences on their potency towards the individual PKC isoforms tested. Furthermore, for some isoforms, they showed potencies even higher than those of the PKC inhibitors used. Therefore, 3,4-dihydroxyxanthone and 1-formyl-4-hydroxy-3-methoxyxanthone can be used as PKC inhibitors for characterization of PKC-mediated effects and for the development of new PKC isoform-selective inhibitors.

Results

Expression of mammalian PKC-α, -βI, -δ, -η and -ζ was confirmed by immunoblotting, of protein extracts of yeast cells, transformed with the gene of a single mammalian PKC isoform, grown in the presence of the transcription inducer (2% galactose). Expression of each of these PKC isoforms resulted in a single antigenic band, which co-migrated with the respective recombinant protein. Protein extracts of transformed yeast cells grown in the absence of galactose did not present antigenic bands (Fig. 1).

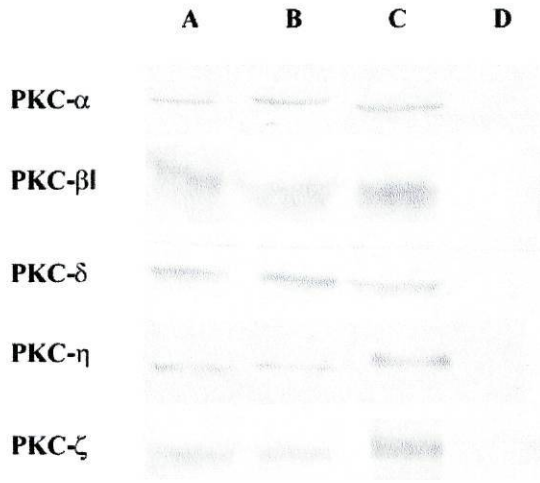


Figure 1. Immunodetection of PKC-α, -βI, -δ, -η and -ζ isoforms expressed in transformed *Saccharomyces cerevisiae* (CG379). Individual immunoblots are presented in a horizontal arrangement and were obtained from proteins extracts (~40 μg protein/lane) from cultures grown in selective medium in the presence of 2% galactose (lanes A and B; duplicate samples) or in the absence of 2% galactose (lane D). Positive controls (lane C; 4 μg) were obtained using recombinant proteins PKC-α (MW 76,799 Da), PKC-βI (MW 76,790 Da), PKC-δ (MW 77,517 Da), PKC-η (MW 77,600 Da) and PKC-ζ (MW 67,740 Da).

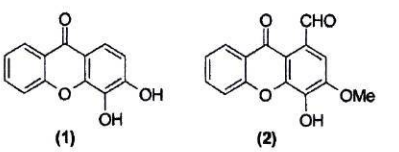


Figure 2. Chemical structure of 3,4-dihydroxyxanthone (**1**) and 1-formyl-4-hydroxy-3-methoxyxanthone (**2**).

Yeast phenotypic assay

PMA (considered a standard activator for the classical and novel PKC isoforms) was tested in yeast expressing PKC-α, -βI, -δ or -η, in concentrations up to 10^{-5} M (higher concentrations were not possible to test due to its low solubility in the culture medium). Since atypical PKC isoforms are not activated by phorbol esters,[25] arachidonic acid (up to 10^{-5} M) was used as the standard activator for PKC-ζ.[26] Effects of drugs were expressed as percentage of the maximal growth inhibition caused by the standard PKC activator (PMA for PKC-α, -βI, -δ, and -η or arachidonic acid for PKC-ζ; 100% growth inhibition was assumed to be that caused by 10^{-5} M of the respective PKC activator).

In the absence of galactose, neither PMA nor arachidonic acid altered yeast growth. The solvent used (DMSO; final concentration 0.1%) did not change yeast growth either in the presence or in the absence of galactose (not shown).

The influence of established PKC inhibitors (chelerythrine and NPC 15437) and of 3,4-dihydroxyxanthone (**1**) and 1-formyl-4-hydroxy-3-methoxyxanthone (**2**) (Fig. 2) on the growth of yeast expressing individual PKC isoforms was also studied. In the absence of galactose, chelerythrine, xanthones **1** and **2**, all tested in the concentration of 10^{-5} M, did not influence yeast growth. NPC 15437, in concentrations higher than 10^{-6} M, inhibited yeast growth. Therefore, to avoid these effects non-mediated by the expressed mammalian PKC isoform, 10^{-6} M was the maximal concentration of NPC 15437 tested when expression of mammalian PKC isoforms were induced (galactose present in the medium).

In the presence of galactose, PMA caused a concentration-dependent inhibition on growth of yeast expres-

Table 1. Yeast growth inhibition caused by 10^{-5} M PMA on the PKC isoforms studied

PKC isoforms	Growth inhibition caused by 10^{-5} M PMA
α	40.6±1.9 (n=36)
βI	36.1±1.1 (n=56)
δ	26.6±0.6 (n=52)
η	21.3±0.7 (n=52)
ζ	0.2±1.4 (n=36)[a]

Growth in the presence of solvent was considered to be 0% growth inhibition (100% growth; see Experimental for details). Each value represents the mean ± SEM of the indicated n determinations.
[a]Growth inhibition caused by 10^{-5} M arachidonic acid was 23.9±0.7 (n=56).

sing cPKCs or nPKCs isoforms. EC_{50} values were (nM) 99.4±10.1 (PKC-α), 214.7±48.0 (PKC-βI), 453.1±32.8 (PKC-δ) and 7.6±0.6 (PKC-η) ($n=64$), for the isoform indicated. In yeast expressing PKC-ζ, arachidonic acid, but not PMA, caused a concentration-dependent growth inhibition of yeast expressing this isoform, with an EC_{50} of 208.2±30.3 nM ($n=64$). Maximal values of growth inhibition caused by 10^{-5} M PMA (or arachidonic acid for PKC-ζ), on the PKC isoforms tested are presented on Table 1.

In the yeast phenotypic assay, a PKC inhibitor may be characterized by its capacity to block the growth inhibition caused by a PKC activator[6,7] and may cause, per se, a stimulation of yeast growth.[6] Therefore, chelerythrine, NPC 15437 and xanthones **1** and **2** were tested alone and combined with the appropriate PKC activator (PMA for PKC-α, -βI, -δ and -η; arachidonic acid for PKC-ζ).

On a first approach, chelerythrine, NPC 15437 and xanthones were tested alone and only on the maximal

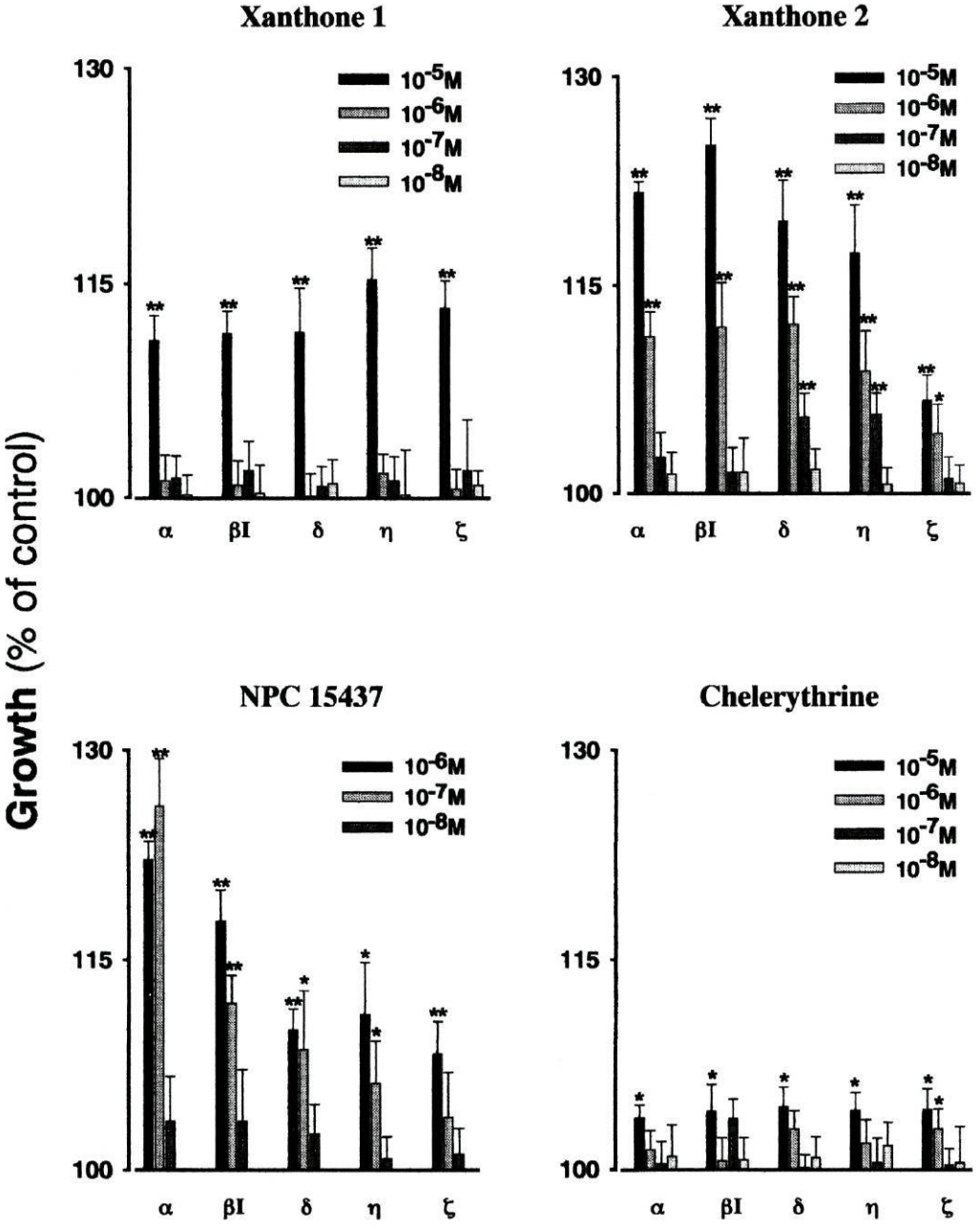


Figure 3. Effects of xanthones **1** and **2** and of the PKC inhibitors (chelerythrine and NPC 15437) on the growth of yeast expressing individual PKC isoforms (α, βI, δ, η or ζ). Yeast cells expressing the indicated mammalian PKC isoform were incubated with each compound at the concentration indicated or solvent (DMSO, 0.1% final concentration). Results are expressed as % of growth. Shown are means ± SEM of 16–24 determinations. Significantly different from growth in the presence of solvent: $*P<0.05$, $**P<0.001$ (paired Student's t test).

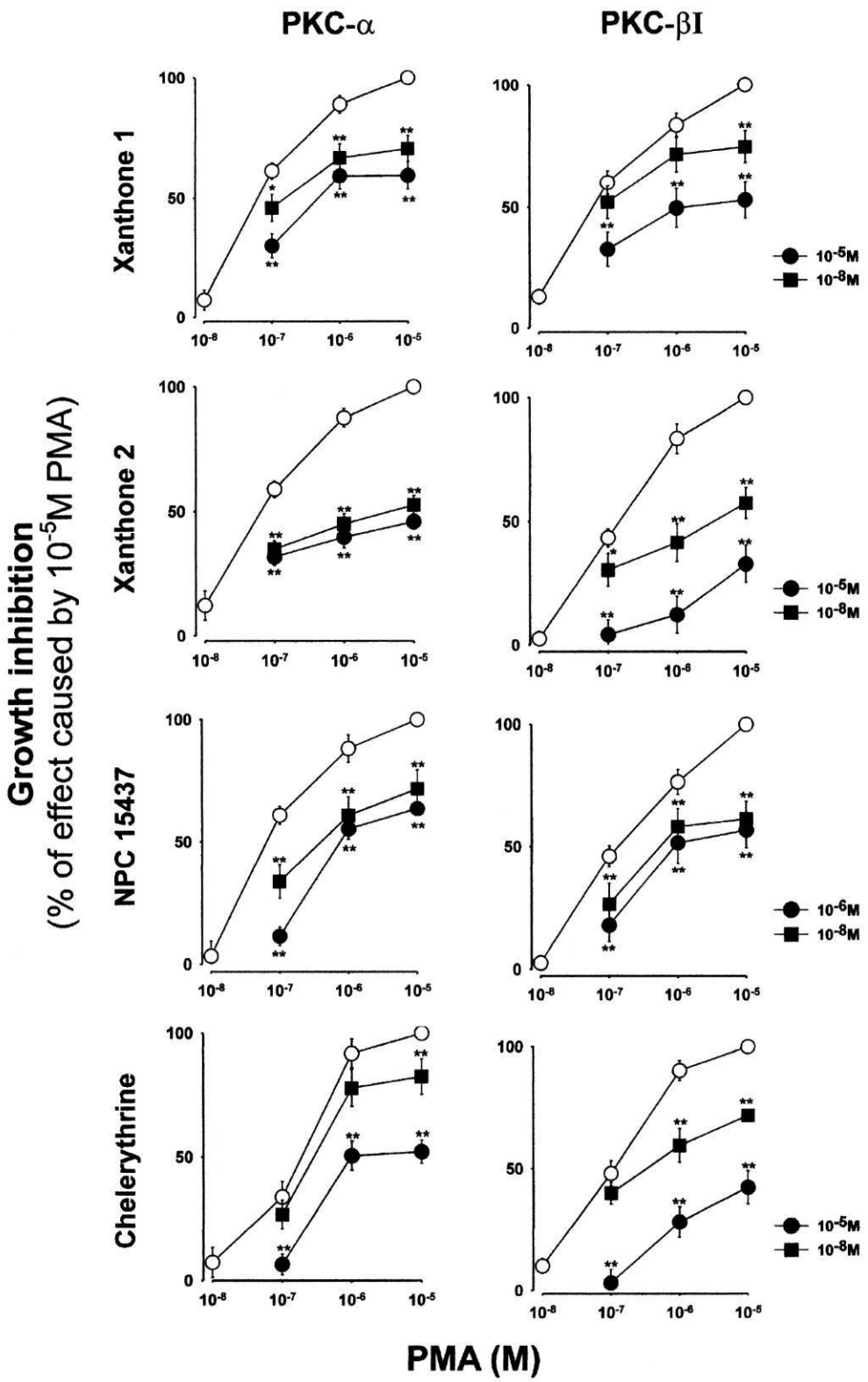


Figure 4. Interaction experiments on cPKCs (α and βI): concentration-response curves for PMA alone (open circles) and in the presence of 10^{-5} M xanthone **1**, xanthone **2**, chelerythrine and 10^{-6} M NPC 15437 (filled circles) or 10^{-8} M (filled squares). Results are expressed as % of the maximal effect caused by 10^{-5} M PMA. Shown are means ± SEM of 16–20 determinations. Significantly different from growth inhibition caused by PMA alone: $^{*}P < 0.05$, $^{**}P < 0.001$ (unpaired Student's *t* test).

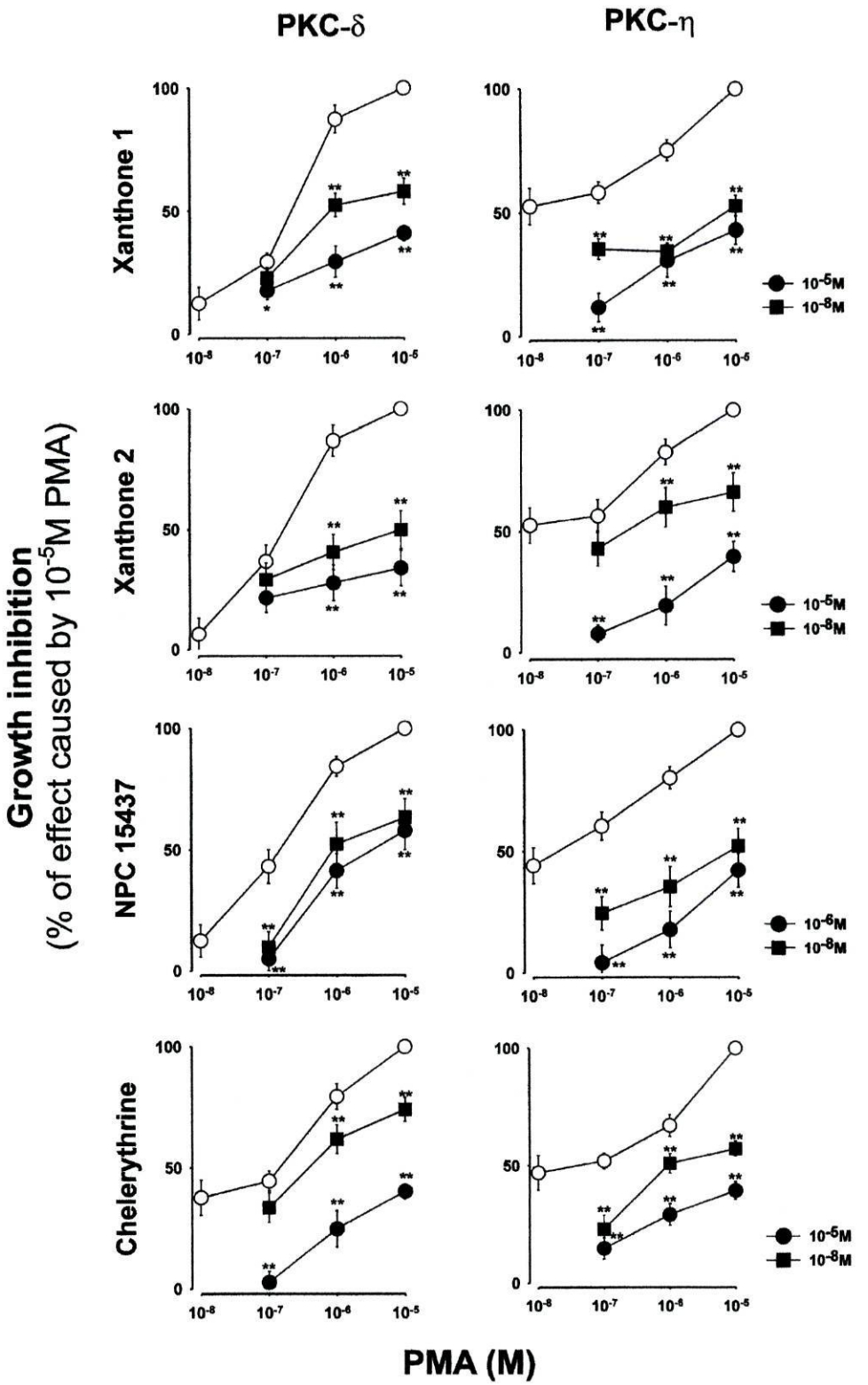


Figure 5. Interaction experiments on nPKCs (δ and η): concentration-response curves for PMA alone (open circles) and in the presence of 10^{-5} M xanthone **1**, xanthone **2**, chelerythrine and 10^{-6} M NPC 15437 (filled circles) or 10^{-8} M (filled squares). Results are expressed as % of the maximal effect caused by 10^{-5} M PMA. Shown are means ± SEM of 16–20 determinations. Significantly different from growth inhibition caused by PMA alone: $^{*}P < 0.05$, $^{**}P < 0.001$ (unpaired Student's *t* test).

concentration feasible under these experimental conditions: 10^{-5} M for chelerythrine and xanthones **1** and **2**; 10^{-6} M for NPC 15437. In general, both PKC inhibitors chelerythrine and NPC 15437, and xanthones **1** and **2** stimulated growth of yeast expressing the individual mammalian PKC isoforms (Fig. 3), although this growth stimulation was less marked with chelerythrine than with NPC 15437 or xanthones **1** or **2**. A decrease on concentration reduced the growth stimulation induced by these drugs. At 10^{-8} M, chelerythrine, NPC 15437, xanthones **1** and **2** failed to cause a significant stimulation of yeast growth (Fig. 3).

On a subsequent series of experiments, the ability of chelerythrine, NPC 15437, xanthones **1** and **2** to block the PKC activator-induced growth inhibition was investigated. These compounds were tested both at the maximal concentration they showed to be selective for PKC (10^{-5} M for chelerythrine and xanthones **1** and **2**; 10^{-6} M for NPC 15437) and at 10^{-8} M (concentration at which all failed to significantly change growth of transformed yeast). Effects of chelerythrine, NPC 15437 and xanthones **1** and **2** on the growth inhibition caused by the PKC activator PMA on yeast expressing PKC-α or -βI and on yeast expressing PKC-δ or -η are shown in Figs. 4 and 5, respectively. Figure 6 shows the effects of chelerythrine, NPC 15437 and xanthones **1** and **2** on the growth inhibition caused by arachidonic acid on yeast expressing PKC-ζ.

In general, both chelerythrine and NPC 15437 reduced the growth inhibition caused by the PKC activator, causing a rightward shift on the concentration-response curve of the PKC activator (compare open and filled symbols in lower panels of Figs. 4–6). The rightward shift was, generally, concentration-dependent being the displacement caused by 10^{-8} M less pronounced than that caused by 10^{-5} M chelerythrine or by 10^{-6} M NPC 15437 (compare filled squares and circles in lower panels of Figs 4–6). Xanthones **1** and **2** also caused, in general, a rightward shift of the concentration-response curve of the PKC activator (see upper panels of Figs 4–6). As observed with chelerythrine and NPC 15437, the rightward shift was, in general, concentration-dependent being the displacement caused by 10^{-8} M less pronounced than that caused by 10^{-5} M xanthones **1** or **2** (compare filled squares and circles in upper panels of Figs 4–6). However, in some cases (xanthone **1** on PKC-ζ, xanthone **2** on PKC-α and-δ, NPC 15437 on PKC-βI and-δ), no concentration-dependence was found, being the rightward shift identical for the highest and the lowest concentration of PKC inhibitor tested ($P > 0.05$; unpaired Student's t test).

In order to compare the potency of the PKC inhibitors to that of xanthones **1** and **2** in blocking the effect of PKC activators, EC_{50} ratios of the PKC activator, obtained in the presence and in the absence of PKC inhibitors or xanthones **1** or **2**, were calculated (EC_{50} is the concentration of the PKC activator that caused half of the growth inhibition caused by 10^{-5} M PMA; arachidonic acid for PKC-ζ). Because the highest concentration of NPC 15437 and xanthones **1** and **2** caused

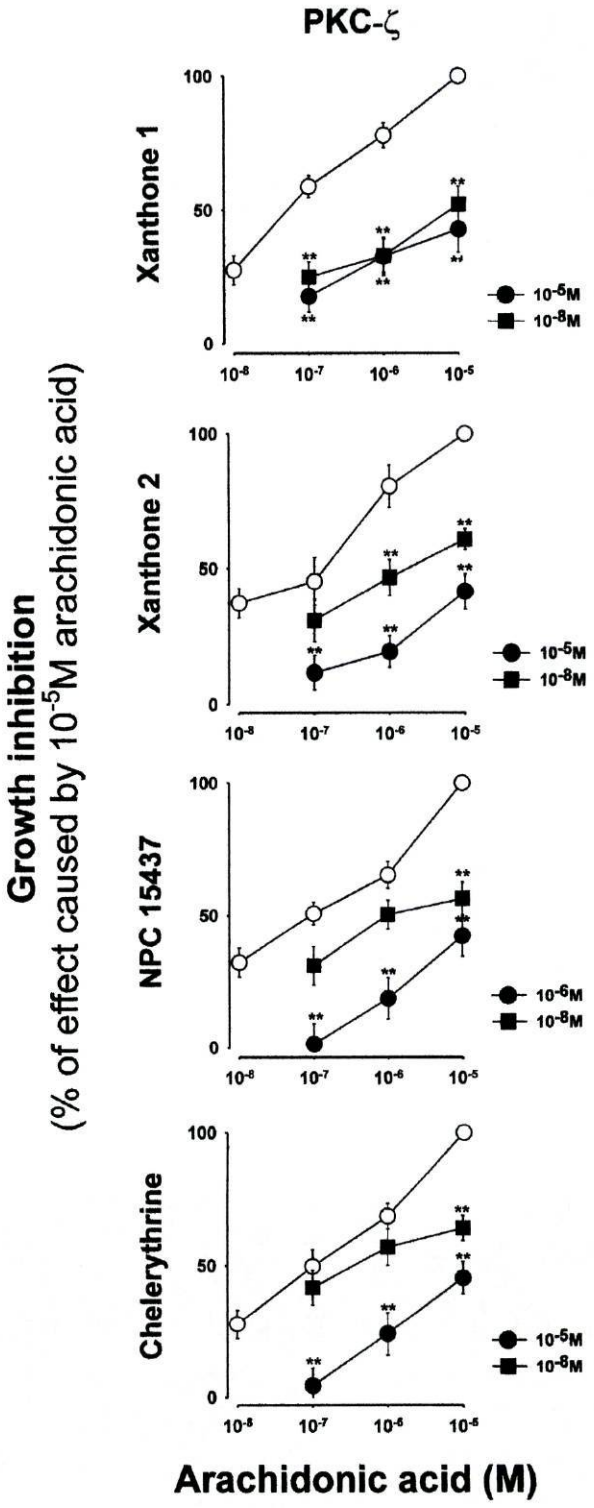


Figure 6. Interaction experiments on aPKC (ζ): concentration–response curves obtained for arachidonic acid alone (open circles) and in the presence of 10^{-5} M xanthone **1**, xanthone **2**, chelerythrine and 10^{-6} M NPC 15437 (filled circles), or 10^{-8} M (filled squares). Results are expressed as % of the maximal effect caused by 10^{-5} M of arachidonic acid. Shown are means ± SEM of 16–20 determinations. Significantly different from growth inhibition caused by arachidonic acid alone: $^{*}P < 0.05$, $^{**}P < 0.001$ (unpaired Student's t test).

Table 2. EC_{50} ratios for xanthones 1 and 2, NPC 15437 and chelerythrine on the individual PKC isoforms tested

Compound	EC_{50} ratio[a]				
	PKC-α	PKC-βI	PKC-δ	PKC-η	PKC-ζ
Xanthone 1	1.8±0.2*§	0.8±0.1*§	4.5±0.8*§	600.0±25.4	40.1±1.8§
Xanthone 2	79.2±6.9	28.7±2.4†	24.0±1.6†	20.0±2.8‡†	10.4±1.1‡†
NPC 15437	5.1±0.5*§	3.7±0.3*§	2.4±0.1*§	556.0±44.7	4.5±0.1‡§
Chelerythrine	1.9±0.3*§	2.9±0.3*§	1.3±0.1*§	63.2±1.7‡	1.4±0.2‡§

The EC_{50} values were considered the concentration of PKC activator that caused half of the growth inhibition caused by 10^{-5} M of PMA (arachidonic acid for PKC-ζ). Shown are means ± SEM of 16–20 determinations. Significant differences: from xanthone 2, $^{*}P < 0.05$; from xanthone 1, $^{\ddagger}P < 0.05$; from PKC-α, $^{\dagger}P < 0.05$; from PKC-η, $^{\S}P < 0.05$ (one way ANOVA, followed by Tukey's post-hoc test).
[a]EC_{50} ratio = EC_{50} (PKC activator + 10^{-8} M compound)/EC_{50} (PKC activator).

growth stimulation, only ratios obtained in the presence of 10^{-8} M (which did not cause a significant stimulation of growth) were used for comparisons (Table 2). According to the EC_{50} ratios obtained, chelerythrine, NPC 15437 and xanthones 1 and 2 presented differences on their potencies towards the PKC isoforms tested and the following order of potencies for the compounds studied was proposed: for chelerythrine, PKC-η > -βI > -α > -ζ = -δ; for NPC 15437, PKC-η > -α = -βI = -ζ > -δ; for xanthone 1, PKC-η > -ζ > -δ > -α = -βI and for xanthone 2, PKC-α > -βI = -δ = -η > -ζ (for definition of the rank order of potency, the signal > was applied only when the EC_{50} ratio for the isoform placed at the left was significantly higher than that of the isoform placed at the right of the signal; otherwise = was applied; $P < 0.05$; unpaired Student's t test). Like NPC 15437 and chelerythrine, xanthone 1 presented an higher EC_{50} ratio on PKC-η. On the other hand, the EC_{50} ratios obtained with xanthone 2 on the different isoforms tested did not differ so markedly, although it was shown to be higher on PKC-α than on other isoforms. Xanthone 1 was the compound which presented the highest EC_{50} ratio on PKC-η and -ζ, whereas xanthone 2 showed the highest EC_{50} ratio on PKC-α, -βI and -δ.

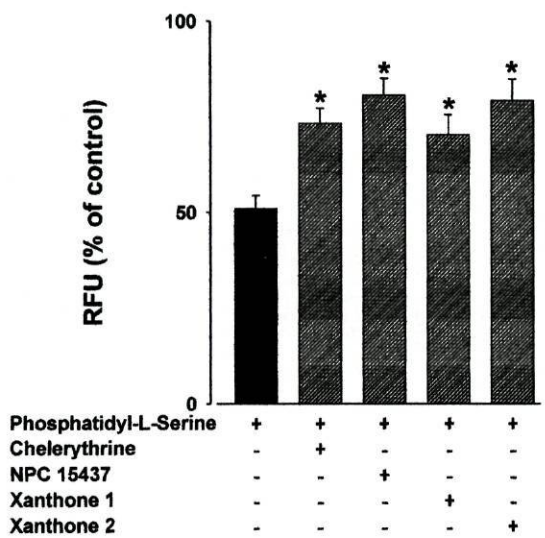


Figure 7. In vitro kinase assay using a purified rat brain PKC enzyme: effects of chelerythrine, NPC 15437, xanthone 1 and xanthone 2 (all tested at the concentration of 10^{-8} M). Relative fluorescence units (RFU) were measured in samples without enzyme (100%; control) or with PKC, which caused a decrease on RFU (see Experimental for details). Phosphatidyl-L-serine, the recommended PKC activator, was present in all samples; drugs or solvent, were tested in the absence or in the presence of PKC. Effects of drugs was expressed as % of RFU obtained in the absence of PKC. Shown are means ± SEM of four assays (8 assays for phosphatidyl-L-serine alone). Significantly different from solvent (phosphatidyl-L-serine alone): $^{*}P < 0.05$ (one way ANOVA, followed by Tukey's post-hoc test).

In vitro kinase assay

To directly test if xanthones 1 and 2 were interacting with PKC, their effects were studied in vitro, on purified rat brain PKC. The test used is based on the ability of PKC inhibitors to revert the decrease on the relative fluorescence units (RFU) caused by the recommended endogenous PKC activator phosphatidyl-L-serine (which is assumed to reflect inhibition of the PKC catalytic activity). Xanthones 1 and 2, chelerythrine and NPC 15437 were used on the lowest concentration tested (10^{-8} M), the same used to obtain the EC_{50} ratios presented on Table 2. Results are shown in Figure 7. Xanthones 1 and 2 were able to revert the effect of the endogenous PKC activator being the effects similar to those caused by chelerythrine and NPC 15437.

Discussion

The yeast phenotypic assay, which uses transformed yeast expressing individual mammalian PKC isoforms, has been proposed as an alternative rapid and simple in vivo assay for the screening of potential PKC modulators. In the present study, the mammalian PKC-α, -βI, -δ, -η or -ζ isoforms were expressed in the same yeast strain. According to previous studies,[21] exposure of transformed yeast expressing a mammalian PKC isoform to a PKC activator, caused a concentration-dependent inhibition of yeast growth, reflecting an increase in the cell doubling time. The inhibition of yeast growth caused by the PKC activator was assumed to be due to its interaction with the PKC isoform expressed, because it did not occur when transformed yeast cells were incubated in medium lacking the expression inducer, galactose. Confirmation that mammalian PKC isoforms were not expressed when galactose was not added to the medium was obtained by immunobloting. PMA failed to influence growth of yeast expressing PKC-ζ, confirming the well known inactivity of phorbol esters on isoforms of the atypical PKC family[25] and provided additional evidence that the growth inhibition caused by

PMA, in this assay, is due to PKC activation. Therefore, in the present experimental conditions, growth inhibition of yeast expressing an individual mammalian PKC isoform caused by the known PKC activators was assumed to reflect PKC activation.

Absolute values of growth inhibition caused by the PKC activators used (PMA or arachidonic acid) differ among the expressed PKC isoforms. These differences may be ascribed to variations on the degree of expression of the mammalian PKC isoform or to differences on the catalytic potential of the expressed isoform. The later possibility is supported by results obtained in vitro that showed that identical amounts of PKC isoforms, in the presence of the same concentration of a PKC activator, led to different degrees of phosphorylation of a given substract.[27] In order to avoid the influence of different absolute values on data interpretation, growth inhibition caused by PKC activators was standardized and, for each isoform, growth inhibition caused by a maximal concentration of PKC activator assumed to be the 100% of growth inhibition reachable under the present experimental conditions and EC_{50} values estimated afterwards.

PKC inhibitors, like chelerythrine and NPC 15437, caused, per se, a stimulation of yeast growth. A similar effect was reported to occur with bryostatin 1 on yeast expressing PKC-γ,[6] an effect probably due to blockade of the PKC activation caused by endogenous activators. Xanthones **1** and **2**, like NPC 15437, also stimulated growth of yeast expressing mammalian PKC isoforms. Nevertheless, chelerythrine, described as a potent PKC inhibitor, only marginally changed growth, what questions the use of growth stimulation as an index of potency for PKC inhibitors. Reasons for the lack of correlation between potency of PKC inhibitors and their ability to increase yeast growth are, at present, not fully understood, but may rely on the different site of interaction between these inhibitors and PKC: NPC 15437 interacts with the C1 region of the regulatory domain[23,24] whereas chelerythrine interacts with the ATP region of the catalytic domain.[22] Xanthones **1** and **2**, when tested alone, also stimulated yeast growth, with a pattern of effects similar to those of NPC 15437. This resemblance may indicate that these xanthones interact with PKC at the same domain of NPC 15437.

Blockade of the PKC activators-induced growth inhibition by established PKC inhibitors has been previously reported to reflect inhibition of the mammalian PKC isoform expressed.[6,7] Under these conditions, PKC inhibitors caused a rightward shift of the concentration-response curve for the PKC activator. The rightward shift was estimated by the ratio between the EC_{50} of the PKC activator obtained in the presence and in the absence of the PKC inhibitor. The obtained EC_{50} ratios were used as an index of the potency of the PKC inhibitor on the mammalian PKC isoform expressed.

Chelerythrine and NPC 15437 blocked the growth inhibition caused by PMA (PKC-α, -βI, -δ and -η) or by arachidonic acid (PKC-ζ). Xanthones **1** and **2** also blocked PKC activation caused by PMA or by arachidonic acid. Comparison of the potency was based on the comparison of EC_{50} ratios caused by concentrations of chelerythrine, NPC 15437, xanthone **1** and xanthone **2** that, per se, did not stimulate yeast growth. Under these conditions xanthone **1** caused a pattern of effects similar to that of NPC 15437: both xanthone **1** and NPC 15437 were very potent upon PKC-η and presented a low potency upon PKC-α, -βI and -δ. Chelerythrine presented also a marked potency on PKC-η, although it was less potent than xanthone **1** and NPC 15437. The high potency of xanthone **1** on PKC-ζ must also be emphasised. On PKC-ζ, xanthone **1** seems to be much more potent than NPC 15437 and chelerythrine. Xanthone **2** did not share the pattern of effects presented by NPC 15437 and chelerythrine, but presented the highest potency on PKC-α, -βI and -δ.

Like other PKC inhibitors, differences on the potency of xanthones **1** and **2** to block the effect of PKC activators may be influenced by their ability to reach their intracellular receptor (PKC) in mammalian cells. The yeast phenotypic assay is an in vivo assay, and factors that influence the access of compounds to intracellular receptors in yeast cells are similar to those present in more complex eukariotic cells. Therefore, the activity showed by xanthones **1** and **2** should reflect their potency in vivo.

Confirmation that, xanthones **1** and **2** blocked the effect of PKC activators on yeast growth by interacting with PKC was obtained using an in vitro kinase assay. At the lowest concentration used on the yeast phenotypic assay, xanthones **1** and **2**, like NPC 15437 and chelerythrine, inhibited the PKC catalytic activity, an effect similar to that caused by the established PKC inhibitors.

Previous studies showed that xanthones may act as PKC activators.[21] The present study extends this observation and shows that xanthone derivatives may also act as potent PKC inhibitors. Given the implication of PKC activation in carcinogenesis and other human diseases, PKC inhibition have become a major research target[28] and xanthone derivatives may become an important family to look for potent and selective PKC inhibitors.

Conclusions

This study shows, by using in vitro and in vivo assays, that simple xanthones can act as inhibitors of mammalian α, βI, δ, η and ζ PKC isoforms. The potencies presented by 3,4-dihydroxyxanthone and 1-formyl-4-hydroxy-3-methoxyxanthone on different PKC isoforms suggest that xanthones may be an important group to look for potent and isoform-selective PKC inhibitors.

Experimental

Chemistry

Purifications of compounds were performed by column chromatography; using Merck silica gel 60 (0.50–0.20 mm)

and preparative thin layer chromatography (tlc), using Merck silica gel 60 (GF_{254}). Melting points were obtained in a Köfler microscope and are uncorrected. IR spectra were recorded on a Perkin Elmer 257 in KBr. ^1H and ^{13}C NMR spectra were taken in $CDCl_3$ or DMSO-d_6 at room temperature, on Bruker AC 200 instrument. Chemical shifts are expressed in δ (ppm) values relative to tetramethylsilane (TMS) as an internal reference. MS spectra were recorded as EI (Electronic Impact) mode on a Hitachi Perkin–Elmer.

The synthesis of the following compounds have been carried out as follow.

3,4-Dihydroxyxanthone (1). This compound was obtained by demethylation of 3,4-dimethoxyxanthone according to the procedure described.[29] Mp >330 °C, chloroform (240–241 °C, ethanol/aqueous;[30] 238–240 °C, methanol[31]); υ_{max} (cm^{-1}) KBr: 3527, 3391, 3088, 1593, 1459, 1340, 1229, 1056, 756; ^1H NMR (DMSO-d_6, 200.13 MHz) δ: 8.14 (1H, dd, J=8.1 and 1.6 Hz, H-8), 7.81 (1H, ddd, J=8.6, 6.9 and 1.7 Hz, H-6), 7.62 (1H, dd, J=8.6 and 0.9 Hz, H-5), 7.56 (1H, d, J=8.6 Hz, H-1), 7.42 (1H, ddd, J=8.1, 6.9 and 0.9 Hz, H-7), 6.93 (1H, d, J=8.6 Hz, H-2); ^{13}C NMR (DMSO-d_6, 50.03 MHz) δ: 175.3 (C-9), 155.5 (C-4b), 151.6 (C-3), 146.4 (C-4a), 134.8 (C-6), 132.7 (C-4), 125.9 (C-8), 124.0 (C-7), 120.8 (C-8a), 118.0 (C-5), 116.6 (C-1), 114.7 (C-9a), 113.2 (C-2); MS m/z (rel int): 230 (3, $[M+2]^{+\cdot}$), 229 (22, $[M+1]^{+\cdot}$), 228 (100, $[M^{+\cdot}]$), 200 (14), 171 (9), 126 (10), 115 (12), 100 (8), 77 (5), 63 (7).

1-Formyl-4-hydroxy-3-methoxyxanthone (2). This compound was obtained according to the procedure described.[32]

General methods for the in vivo yeast phenotypic assay

Yeast nitrogen base was from DIFCO (Merck Portugal, Lisboa, Portugal). The kit for protein quantification was from Pierce (Biocontec, Lisboa, Portugal). The secondary alkaline phosphatase-conjugated anti-rabbit IgG detection kit (AP-10), recombinant proteins PKC-α (PK11), PKC-βI (PK16), PKC-δ (PK31), PKC-η (PK46) and PKC-ζ (PK41) were from Oxford Biomedical Research (LabClinics, Barcelona, Spain). Nitrocellulose membranes and all the reagents for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and immunoblots were from BioRad (PACI, Lisboa, Portugal). Acid-washed glass beads, antibodies to PKC-α, PKC-βI, PKC-δ, PKC-η and PKC-ζ, aprotinin, arachidonic acid sodium salt, chelerythrine cloride, *R*-2,6-diamino-*N*-[[1-(1-oxotridecyl)-2-piperidinyl]methyl]-hexanamide dihydrochloride (NPC 15437 dihydrochloride), leupeptin, pepstatin A, phenylmethylsulfonyl fluoride, phorbol 12-myristate 13-acetate (PMA) were from Sigma Aldrich (Sintra, Portugal). All other chemicals used were of analytical grade.

Yeast transformation and cell cultures. Constructed yeast expression plasmids YEp52 and YEp51, encoding the cDNA for bovine PKC-α and for rat PKC-βI, respectively (kindly offered by Dr. Heimo Riedel, Wayne University, Detroit, USA) and YEplac181, encoding the cDNA for the rat PKC-δ, mouse PKC-η or PKC-ζ (kindly offered by Dr. Nigel Goode, Royal Veterinary College, London, UK) were amplified in *Escherichia coli* DH5α and confirmed by restriction analysis. The plasmids used contain galactose-inducible transcriptional elements and the leu2 gene for selection. *Saccharomyces cerevisiae* (*S. cerevisiae*; strain CG379; α *ade5 his7-2 leu2-112 trp1-289α ura3-52 [Kil-O];* Yeast Genetic Stock Center, University of California, Berkeley, USA) was transformed using the lithium acetate method.[33] To ensure the selection of transformed yeast, cells were grown in leucine-free medium, in 1.5% agar plates, at 30 °C.

For the yeast phenotypic assay, transformed cells were incubated in leucine free-medium, with slow shaking, at 30 °C. The leucine free-medium contained 0.7% yeast nitrogen base, 2% glucose (w/v) or the indicated carbon source, amino acids, purines and pyrimidines, according to the transformed yeast requirements. Galactose (2%; w/v), instead of glucose, was included to induce transcription of the mammalian PKC gene.

Cell lysis and immunoblotting. Cell lysis was performed basically as described.[21] The protein concentration was determined using a kit for protein quantification (*Coomassie® Protein Assay Reagent Kit*, Pierce, Biocontec, Lisbon, Portugal). Similar amounts of protein (~40 μg) from protein extracts were then separated on 10% SDS-polyacrylamide gels (Mini-Protean II, BioRad, Hercules, CA, USA). Positive controls (4 μg) were obtained using recombinant proteins PKC-α (MW 76,799 Da), PKC-βI (MW 76,790 Da), PKC-δ (MW 77,517 Da), PKC-η (MW 77,600 Da) and PKC-ζ (MW 67,740 Da). Proteins were electrophoretically transferred to nitrocellulose membranes and probed on immunoblots with specific rabbit antibodies to the individual mammalian PKC isoforms and revealed with a secondary alkaline phosphatase-conjugated anti-rabbit IgG (AP-10, Oxford Biomedical Research, LabClinics, Barcelona, Spain).

Yeast phenotypic assay. Transformed yeast cultures were incubated in leucine-free medium. Optical density values, measured at 620 nm (OD_{620}; Cary 1E Varian spectrophotometer, Palo Alto, CA, USA), were used as an indicator of growth. Transformed yeast were grown to an OD_{620} of approximately 1, collected by centrifugation and diluted to an OD_{620} of 0.05, in medium containing 2% (w/v) galactose (gene transcription-inducer) and 3% (v/v) glycerol (alternative carbon source). Diluted cultures (200 μL) were transferred to 96-wells microtitre plates and incubated for up to 100 h, with slow shaking at 30 °C, either in the presence of drugs or solvent (DMSO 0.1%; final concentration). Growth was monitored by determining the OD_{620} using a plate reader (BioRad Benchmark Microplate Reader; Hercules, CA, USA). In preliminary experiments, growth curves for individual isoforms were determined and the duration of the logarithmic and stationary phases identified. Estimation of drug effects was based on OD_{620} measurements at fixed time points (at 65 h for

cPKC isoforms or at 48 h incubation for nPKC and aPKC), times occurring during the respective logarithmic phase and where a 'steady-state growth inhibition' (period of time during which maximal inhibition of growth was reached and remained constant or changed only slightly) was reached. In individual experiments, OD_{620} was routinely monitored for up to 100 h to confirm whether the 'fixed time points' chosen were appropriate for all the compounds studied (PMA or arachidonic acid). The difference between the maximal OD_{620} reached and that measured at the beginning of incubation was used as an index of yeast growth. Drugs or solvent were added at the beginning and kept throughout the incubation. Yeast growth in the presence of drugs was expressed as percentage of growth observed in parallel experiments in the presence of solvent; it was further transformed into growth inhibition by subtracting that value from 100. Because growth inhibition caused by a maximal concentration of the standard PKC activator varied between isoforms, 100% growth inhibition was assumed to be that caused by 10^{-5} M PMA (or arachidonic acid for PKC-ζ), in order to standardize the maximal inhibition reachable; 0% growth inhibition would occur when growth in the presence of drugs was identical to that in the presence of solvent. Effects of PKC activators were expressed as percentage of that effect.

For interaction experiments, a single concentration of PKC inhibitors or xanthones **1** or **2** were added to PMA (10^{-8}–10^{-5} M; arachidonic acid for PKC-ζ). Concentration-response curves for the PKC activator alone in the absence or in the presence of PKC inhibitors or xanthones **1** or **2**, were obtained and the concentration of PKC activator that caused 50% growth inhibition (EC_{50}) determined. For each compound the EC_{50} ratios [EC_{50} (PKC activator + 10^{-8} M compound)/EC_{50} (PKC activator)] were calculated on each PKC isoform tested.

In vitro kinase assay

The direct interaction between the compounds tested (PKC inhibitors and xanthones **1** and **2**) with PKC was studied in vitro using the IQ™ PKC Assay Kit—Pseudosubstrate Peptide Substrate from Pierce Biotechnology (PACI, Lisboa, Portugal). The assay was performed in agreement with the kit procedure for 96-well plate format. Xanthones **1** and **2**, and the standard PKC inhibitors (chelerythrine and NPC 15437) were tested at the concentration of 10^{-8} M. Purified PKC enzyme standard from rat brain (containing a mixture of α, β and γ isoforms) was from Pierce Biotechnology (PACI, Lisboa, Portugal); it was used at the final concentration of 0.01 Units. Reaction mixtures containing the endogenous PKC activator phosphatidyl-L-serine, were incubated for 90 min and fluorescence intensity determined using a microplate spectrofluorometer SPECTRAmax®GEMINI XS (Molecular Devices Corporation, Sunnyvale, CA, USA). In the presence of PKC, there was a decrease on relative fluorescence units (RFU), reflecting a phosphorylation of the fluorescent substrate. In the presence of PKC inhibitors, substrate phosphorylation is reduced causing an increase on RFU. RFU in samples containing no PKC were considered as 100%. Effects of drugs were expressed as percentage of that value.

Statistical analysis. Results are given as arithmetic means ± SEM and *n* represents the number of determinations. Differences between means were tested for significance using either paired Student's *t* test, unpaired Student's *t* test or one way ANOVA, followed by Tukey's post-hoc test. A *P* value less than 0.05 was taken to be statistically significant.

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ANEXO VIII

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Inhibition of α, βI, δ, η and ζ Protein Kinase C Isoforms by Xanthonolignoids

LUCÍLIA SARAIVA[a,b], PAULA FRESCO[a], EUGÉNIA PINTO[b], EMÍLIA SOUSA[c], MADALENA PINTO[c] and JORGE GONÇALVES[a,*]

[a]*Serviço de Farmacologia, CEQOFFUP, Faculdade de Farmácia, Universidade do Porto, rua Aníbal Cunha, 164, 4050-047 Porto, Portugal;*
[b]*Serviço de Microbiologia, CEQOFFUP, Faculdade de Farmácia, Universidade do Porto, rua Aníbal Cunha, 164, 4050-047 Porto, Portugal;*
[c]*Serviço de Química Orgânica, CEQOFFUP, Faculdade de Farmácia, Universidade do Porto, rua Aníbal Cunha, 164, 4050-047 Porto, Portugal*

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The effect of the xanthonolignoids *trans*-(±)-kielcorin C, *cis*-(±)-kielcorin C, *trans*-(±)-kielcorin D, *trans*-(±)-isokielcorin D and *trans*-(±)-kielcorin E on isoforms α, βI, δ, η and ζ of protein kinase C (PKC) was studied using the yeast phenotypic assay.

All the compounds tested revealed an effect compatible with PKC inhibition, similar to that exhibited by the well established PKC inhibitor chelerythrine, and with differences in their potency towards the distinct isoforms tested, being, in general, potent inhibitors of the atypical PKC isoform (PKC-ζ). PKC inhibition caused by these kielcorins was confirmed using an *in vitro* kinase assay.

The present study constitutes the first attempt to unravel the molecular mechanism of kielcorins activity, and shows that xanthonolignoids are a promising group of compounds to investigate for isoform selective PKC inhibitors.

Keywords: Protein kinase C; PKC isoforms; Yeast phenotypic assay; PKC inhibitors; Xanthonolignoids; Kielcorins

INTRODUCTION

The ubiquitous enzyme protein kinase C (PKC) is a family of serine-threonine kinases with pivotal roles in cellular regulation, signal transduction and neoplastic promotion.[1,2] PKC isoforms are grouped into at least three groups: the classical PKCs (cPKCs), which include the isoforms α, βI, βII and γ; the novel PKCs (nPKCs), which include the isoforms δ, ε, θ, and η; and the atypical PKCs (aPKCs), which include the isoforms ζ and λ/ι.[2] More recently, a fourth group of structurally distinct PKCs has been identified, the so-called PKC-related kinases (PRKs).[2] A new isoform, PKCμ/PKD, has also been reported, but its inclusion in the PKC family remains controversial.[2]

Clarification of the role of individual PKC isoforms in the cell has been made difficult by the lack of selective PKC activators and inhibitors,[3] which has been hampered by methodological limitations, caused by the coexistence of several PKC isoforms. These limitations can be circumvented by using the yeast phenotypic assay,[4,5] which uses yeast expressing an individual mammalian PKC isoform. The assay is based on the growth inhibition of transformed yeast (reflecting an increase in the cell doubling time), which is proportional to the degree of PKC activation.[6,7] Because only one mammalian PKC isoform is functionally expressed, separately, and effects non-mediated by that isoform can be easily detected by measuring growth in the absence of expression, it represents a convenient method to screen potential PKC activators and inhibitors and to elucidate their isoform selectivity. In the yeast phenotypic assay, PKC activators cause a concentration-dependent growth inhibition of transformed yeast,[6,7] while PKC inhibitors block that growth inhibition.[8,9] Therefore, the potency of PKC activators can be estimated by the concentration that cause half of the maximal growth inhibition attainable by PKC activators (EC_{50}) and the potency of PKC inhibitors by the increase in the EC_{50} value caused by the PKC inhibitor [EC_{50} ratio = EC_{50}(PKC activator + PKC inhibitor)/EC_{50} (PKC activator)].

*Corresponding author. Tel.: +351 22 2078932. Fax: +351 22 2078969. E-mail: jorge.goncalves@ff.up.pt

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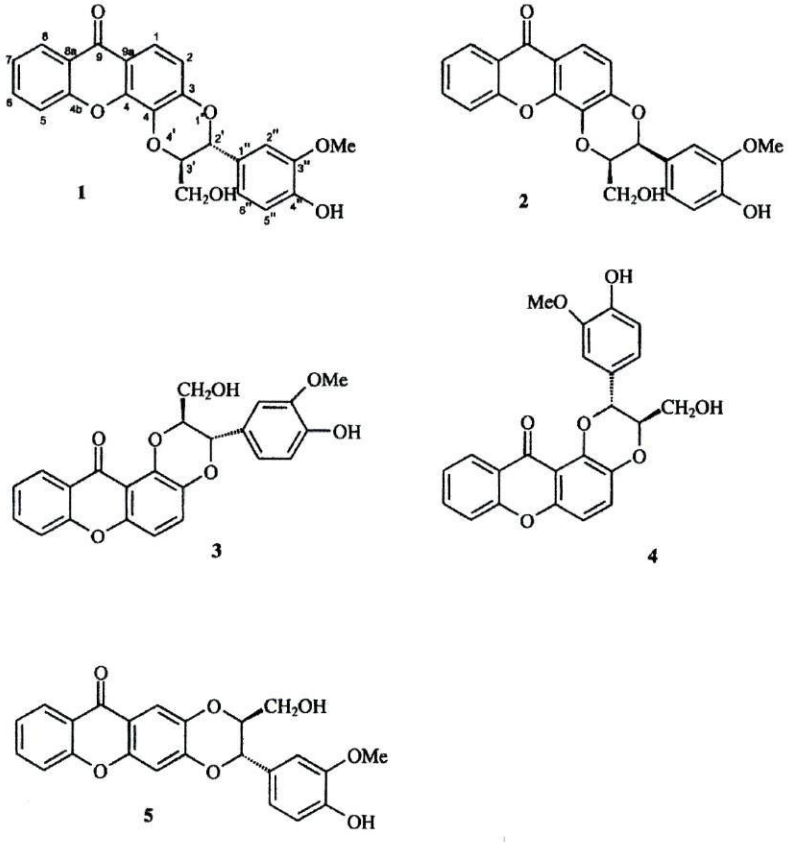


FIGURE 1 Chemical structures of *trans*-(±)-kielcorin C (**1**), *cis*-(±)-kielcorin C (**2**), *trans*-(±)-kielcorin D (**3**), *trans*-(±)-isokielcorin D (**4**) and *trans*-(±)-kielcorin E (**5**).

Xanthone derivatives have been reported to mediate several important biological activities.[10,11,12] Recent studies suggested that these compounds may act, at least in part, by interacting with PKC, causing effects compatible with either PKC activation[4] or PKC inhibition.[5]

Kielcorins are 1,4-benzodioxane derivatives belonging to the xanthonolignoids family, with a skeleton in which a phenylpropane unit is linked to a xanthone nucleus through a dioxane bridge.[10,13,14] Recent studies showed that kielcorins have an hepatoprotective effect[15] and an antiproliferative effect against three human tumor cell lines: MCF-7 (breast), TK-10 (renal) and UACC-62 (melanoma).[13,14] However, to our knowledge, no data is available about the molecular basis of the biological activity of kielcorins. Previously, the biomimetic synthesis of natural kielcorin and kielcorin B was achieved.[16,17] In order to obtain new compounds belonging to this family, synthesis of *trans*-(±)-kielcorin C (**1**), *cis*-(±)-kielcorin C (**2**), *trans*-(±)-kielcorin D (**3**), *trans*-(±)-isokielcorin D (**4**) and *trans*-(±)-kielcorin E (**5**) (Figure 1) was undertaken.[13] The synthetic approach of these constitutional isomers was based on the biomimetic pathway, by oxidative coupling of coniferyl alcohol (**6**) with an appropriate xanthone (**7**)[13] (Figure 2).

In the present study, the effects of *trans*-(±)-kielcorin C (**1**), *cis*-(±)-kielcorin C (**2**), *trans*-(±)-kielcorin D (**3**), *trans*-(±)-isokielcorin D (**4**) and *trans*-(±)-kielcorin E (**5**) on PKC isoforms α and βI (cPKCs), δ and η (nPKCs) and ζ (aPKCs) were

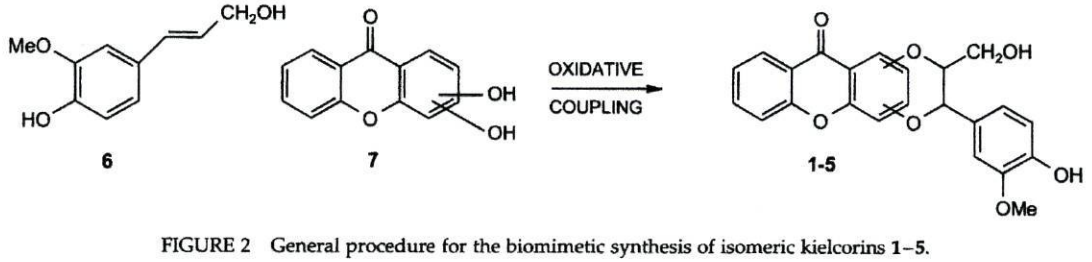


FIGURE 2 General procedure for the biomimetic synthesis of isomeric kielcorins **1–5**.

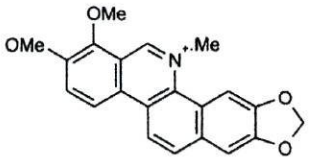


FIGURE 3 Chemical structure of chelerythrine.

characterized in our laboratory using the yeast phenotypic assay. The inhibition of PKC by these five isomeric kielcorin derivatives is reported and their effects and structures were compared to those of the PKC inhibitor chelerythrine[18] (Figure 3). Differences in their potency towards the individual PKC isoforms tested were also detected.

This work represents the first molecular approach for the study of the biological effects of kielcorins and reveals that they act as PKC inhibitors.

MATERIALS AND METHODS

Synthesis

Purification of compounds was performed by column chromatography, using Merck sílica gel 60 (0.50–0.20 mm), flash chromatography [Merck silica gel 60 (0.040–0.063 mm)] and preparative thin layer chromatography (tlc), employing Merck sílica gel 60 (GF_{254}). M.p. were determined on a *Köfler microscope* and are uncorrected, IR Spectra on a *Perkin Elmer 257* spectrophotometer with KBr microplates, UV Spectra on a *Varian CARY 1E* spectrophotometer, ^{1}H and ^{13}C-NMR Spectra on a *Bruker AC 200, DRX 300,* or *DRX 500* spectrometer at r.t., in $CDCl_3$ or (D_6)DMSO with δ in ppm relative to $SiMe_4$ as an internal reference and coupling constants *J* in Hz. ^{1}H-NMR assignments were made using 2D COSY experiments, while ^{13}C-NMR assignments were made using one-dimensional selective INEPT and 2D HETCOR, HSQC and HMBC experiments (long range C/H coupling constants were optimised to 7 and 1 Hz). NOE experiments were determined by means of NOE difference technique, using an irradiation time of 2 s and a relaxation delay of 4 s. EI-MS Spectra: *Hitachi Perkin-Elmer RMU-6M* spectrometer, in *m/z*. HR-MS Spectra: *VG AutoSpec-Q* spectromer; recorded as EI (electronic impact) mode.

General Procedure for the Synthesis of Kielcorin Derivatives

The appropriate dihydroxyxanthone (**7**, 615 mg, 2.7 mmol) and coniferyl alcohol (**6**, 500 mg, 2.7 mmol) in a mixture of 1:1 toluene:acetone were stirred in the presence of silver oxide (860.6 mg, 3.1 mmol) in the dark for 2–3 days. The suspension was filtered, the filtrate evaporated, and the crude product purified by column chromatography using mixtures of hexane:chloroform (20:80 for compounds **1**, **2**, and **5**) and chloroform:methanol (98:2 for compounds **3** and **4**). The isolation of the components **1–2**, **3–4** and **5** was then carried out by preparative tlc ($CHCl_3$:MeOH 95:5).

trans-(±)-Kielcorin C (1)

M.p. 243–247° (Me_2CO). UV (EtOH): 309, 283, 239, 204 nm. IR (KBr): 3398, 1642, 1605, 1562, 1449, 1338, 1264, 1025, 861 cm^{-1}. ^{1}H-NMR (300.13 MHz, (D_6)DMSO) δ: 9.25 (*s*, *HO*-C(4″)), 8.20 (*dd*, *J* = 7.7, and 1.6, H-C(8)), 7.87 (*ddd*, *J* = 8.3, 7.4, and 1.6, H-C(6)), 7.70 (*d*, *J* = 8.8, H-C(1)), 7.69 (*d*, *J* = 8.3, H-C(5)), 7.49 (*ddd*, *J* = 7.7, 7.4, and 0.7, H-C(7)), 7.07 (*d*, *J* = 8.8, H-C(2)), 7.07 (*d*, *J* = 1.7, H-C(2″)), 6.92 (*dd*, *J* = 8.1 and 1.7, H-C(6″)), 6.83 (*d*, *J* = 8.1, H-C(5″)), 5.15 (*d*, *J* = 7.9, H-C(2′)), 5.13 (*t*, *J* = 6.1, CH_2OH), 4.36–4.40 (*m*, H-C(3′)), 3.79 (*s*, MeO-C(3″)), 3.71–3.76 (*m*, CH_2OH), 3.40–3.50 (*m*, CH_2OH). ^{13}C-NMR (75.47 MHz, (D_6)DMSO): 175.1 (C(9)), 155.4 (C(4b)), 148.8 (C(3)), 147.7 (C(3″)), 147.2 (C(4″)), 145.9 (C(4a)), 135.2 (C(6)), 131.8 (C(4)), 126.7 (C(1″)), 126.0 (C(8)), 124.5 (C(7)), 121.0 (C(8a)), 120.7 (C(6″)), 118.1 (C(5)), 117.5 (C(1)), 115.7 (C(9a)), 115.4 (C(5″)), 113.9 (C(2)), 111.8 (C(2″)), 78.0 (C(3′)), 76.5 (C(2′)), 59.9 (CH_2OH), 55.7 (*Me*O-C(3″)). EI-MS: 406 (6, M^+), 356 (26), 341 (22), 327 (17), 306 (18), 292 (12), 281 (100), 261 (38), 253 (5), 228 (13), 215 (8), 180 (10), 165 (8), 141 (10), 115 (18), 105 (9), 91 (17), 77 (18), 63 (10), 55 (13). HRMS for $C_{23}H_{19}O_7$: Calc. 407.1131; Found: 407.1130.

cis-(±)-Kielcorin C (2)

M.p. 200–202° (Me_2CO). UV (EtOH): 308, 283, 239, 204 nm. IR (KBr): 3420, 1644, 1607, 1511, 1454, 1379, 1267, 1100, 860 cm^{-1}. ^{1}H-NMR (300.13 MHz, (D_6)DMSO) δ: 9.09 (*s*, *HO*-C(4″)), 8.21 (*dd*, *J* = 7.6 and 1.6, H-C(8)), 7.83 (*ddd*, *J* = 8.0, 7.5, and 1.6, H-C(6)), 7.75 (*d*, *J* = 8.7, H-C(1)), 7.67 (*d*, *J* = 8.0, H-C(5)), 7.46 (*dd*, *J* = 7.6 and 7.5, H-C(7)), 7.05 (*d*, *J* = 8.8, H-C(2)), 6.99 (br. *s*, H-C(2″)), 6.95–6.74 (*m*, H-C(5″) and H-C(6″)), 5.46 (*d*, *J* = 2.8, H-C(2′)), 4.98 (*t*, *J* = 5.4, CH_2OH), 4.63–4.68 (*m*, H-C(3′)), 3.83 (*s*, MeO-C(3″)), 3.42–3.61 (*m*, CH_2OH). ^{13}C-NMR (75.47 MHz, (D_6)DMSO): 175.5 (C(9)), 155.9 (C(4b)), 148.7 (C(3)), 147.8 (C(3″)), 147.1 (C(4″)), 146.7 (C(4a)), 135.2 (C(6)), 131.1 (C(4)), 126.7 (C(1″)), 126.4 (C(8)), 124.6 (C(7)), 121.5 (C(8a)), 119.5 (C(6″)), 118.4 (C(5)), 118.2 (C(1)), 116.4 (C(9a)), 115.9 (C(5″)), 114.1 (C(2)), 111.4 (C(2″)), 77.8 (C(3′)), 76.4 (C(2′)), 58.6 (CH_2OH), 56.0 (*Me*O-C(3″)). EI-MS: 406 (19, M^+), 281 (1.8), 246 (24), 184 (14), 153 (100), 149 (8), 137 (17), 111 (1.3), 93 (60), 83 (22), 72 (8), 65 (38), 59 (14), 57 (41). HRMS for $C_{23}H_{19}O_7$: Calc. 407.1131; Found: 407.1131.

***trans*-(±)-Kielcorin D (3)**

M.p. 258–260° (Me_2CO). UV (EtOH): 372, 278, 259, 239, 237, 212 nm. IR (KBr): 3498, 1635, 1602, 1309, 1270, 1228, 1047, 762 cm^{-1}. ^{1}H-NMR (300.13 MHz, (D_6)DMSO) δ: 9.20 (*s*, *HO*-C(4″)), 8.04 (*dd*, J = 7.9 and 1.6, H-C(8)), 7.80 (*ddd*, J = 8.1, 7.8, and 1.6, H-C(6)), 7.56 (*d*, J = 8.1, H-C(5)), 7.45 (*d*, J = 9.1, H-C(3)), 7.40 (*dd*, J = 7.9 and 7.8, H-C(7)), 7.14 (*d*, J = 9.1, H-C(4)), 7.10 (*d*, J = 1.7, H-C(2″)), 6.92 (*dd*, J = 8.1 and 1.7, H-C(6″)), 6.83 (*d*, J = 8.1, H-C(5″)), 5.05 (*d*, J = 7.6, H-C(3′)), 5.05 (*t*, J = 7.6, CH_2OH), 4.20–4.23 (*m*, H-C(2′)), 3.78 (*s*, MeO-C(3″)), 3.57–3.62 (*m*, CH_2OH), 3.41 (under H_2O signal, CH_2OH). ^{13}C-NMR (75.47 MHz, (D_6)DMSO): 174.9 (C(9)), 154.6 (C(4b)), 150.9 (C(4a)), 147.6 (C(3″)), 147.0 (C(4″)), 143.1 (C(1)), 138.9 (C(2)), 134.8 (C(6)), 127.1 (C(1″)), 125.9 (C(8)), 124.0 (C(7)), 123.7 (C(3)), 121.7 (C(8a)), 120.5 (C(6″)), 117.6 (C(5)), 115.3 (C(5″)), 111.92 and 111.86 (C(9)a and C(2″)), 109.5 (C(4)), 77.2 (C(2′)), 76.1 (C(3′)), 60.0 (CH_2OH), 55.7 (*Me*O-C(3″)). EI-MS: 406 (6, M^+), 369 (2), 327 (7), 285 (6), 228 (14), 184 (22), 153 (100), 137 (10), 125 (13), 110 (9), 93 (61), 81 (9), 65 (35). HRMS for $C_{23}H_{19}O_7$: Calc. 407.1131; Found: 407.1130.

***trans*-(±)-Isokielcorin D (4)**

M.p. 136–139° (Me_2CO). UV (EtOH): 374, 280, 260, 240, 209 nm. IR (KBr): 3436, 1626, 1520, 1459, 1383, 1319, 1269, 1110, 1060, 1028, 809 cm^{-1}. ^{1}H-NMR (500.13 MHz, (D_6)DMSO) δ: 9.20 (*s*, *HO*-C(4″)), 8.12 (*dd*, J = 7.8 and 1.4, H-C(8)), 7.81 (*ddd*, J = 8.1, 7.4, and 1.4, H-C(6)), 7.57 (*d*, J = 8.1, H-C(5)), 7.43 (*d*, J = 9.1, H-C(3)), 7.43 (*dd*, J = 7.8 and 7.4, H-C(7)), 7.12 (*d*, J = 9.1, H-C(4)), 7.04 (*d*, J = 1.4, H-C(2″)), 6.89 (*d*, J = 8.1 and 1.4, H-C(6″)), 6.80 (*d*, J = 8.1, H-C(5″)), 5.00 (*d*, J = 7.8, H-C(2′)), 4.99 (*t*, J = 7.6, CH_2OH), 4.31–4.34 (*m*, H-C(3′)), 3.77 (*s*, MeO-C(3″)), 3.71–3.73 (*m*, CH_2OH), 3.41 (under H_2O signal, CH_2OH). ^{13}C NMR (75.47 MHz, (D_6)DMSO): 174.6 (C(9)), 154.5 (C(4b)), 150.0 (C(4a)), 147.7 (C(3″)), 147.3 (C(4″)), 143.2 (C(1)), 139.2 (C(2)), 135.0 (C(6)), 127.3 (C(1″)), 126.2 (C(8)), 123.8 (C(3)), 123.7 (C(7)), 122.3 (C(8a)), 120.8 (C(6″)), 117.7 (C(5)), 115.1 (C(5″)), 111.7 (C(9a) and C(2″)), 107.9 (C(4)), 78.0 (C(3′)), 75.2 (C(2′)), 59.4 (CH_2OH), 55.4 (*Me*O-C(3″)). EI-MS: 406 (50, M^+), 239 (7), 228 (100), 199 (25), 180 (73), 170 (11), 153 (18), 137 (64), 124 (44), 103 (5), 91 (16), 77 (14), 65 (10). HRMS for $C_{23}H_{19}O_7$: Calc. 407.1131; Found: 407.1131.

***trans*-(±)-Kielcorin E (5)**

M.p. 247–249° (Me_2CO). UV (EtOH): 353, 307, 277, 241 nm. IR (KBr): 3498, 1635, 1602, 1309, 1270, 1228, 1047, 762 cm^{-1}. ^{1}H-NMR (300.13 MHz, (D_6)DMSO) δ: 9.25 (*s*, *HO*-C(4″)), 8.17 (*dd*, J = 7.7 and 1.2, H-C(8)), 7.84 (*ddd*, J = 8.0, 7.6, and 1.2, H-C(6)), 7.62 (*d*, J = 8.0, H-C(5)), 7.61 (*s*, H-C(1)), 7.46 (*ddd*, J = 7.7 and 7.5, H-C(7)), 7.23 (*s*, H-C(4)), 7.06 (*d*, J = 1.3, H-C(2″)), 6.90 (*dd*, J = 8.1 and 1.3, H-C(6″)), 6.82 (*d*, J = 8.1, H-C(5″)), 5.13 (*d*, J = 8.1, H-C(3′)), 5.06 (br. *s*., CH_2OH), 4.33–4.35 (*m*, H-C(2′)), 3.78 (*s*, MeO-C(3″)), 3.58 (br. *d*, J = 11.4, CH_2OH), 3.38 (under H_2O signal, CH_2OH). ^{13}C-NMR (75.47 MHz, (D_6)DMSO): 174.8 (C(9)), 155.7 (C(4b)), 151.1 (C(3)), 150.6 (C(4a)), 147.7 (C(3″)), 147.3 (C(4″)), 141.3 (C(2)), 135.0 (C(6)), 126.5 (C(1″)), 125.8 (C(8)), 124.1 (C(7)), 120.8 (C(6″)), 120.6 (C(8a)), 118.0 (C(5)), 115.4 (C(5″) and C(9a)), 111.9 (C(2″)), 111.2 (C(1)), 104.8 (C(4)), 77.8 (C(2′)), 77.0 (C(3′)), 60.0 (CH_2OH), 55.7 (*Me*O-C(3″)). EI-MS: 406 (0.02, M^+), 369 (0.8), 327 (6), 310 (0.8), 285 (6), 267 (0.6), 260 (1.4), 246 (1), 215 (2), 208 (0.6), 184 (21), 153 (100), 137 (3), 125 (12), 110 (9), 93 (62), 81 (8), 65 (35). HRMS for $C_{23}H_{19}O_7$: Calc. 407.1131; Found: 407.1131.

Molecular Calculations

All calculations were performed with *Gaussian 98*.[19] The geometry optimisation was performed using the AM1 semi-empirical method. The optimised conformations were submitted to a single point calculation, using the density functional theory, with the B3LYP functional at 3-21G basis set. The potential isosurfaces were obtained using the cubegen utility present in the *Gaussian* package. All visualizations were obtained with *Molekel 4.2*.[20]

General Methods for the Yeast Phenotypic Assay

Yeast nitrogen base was from DIFCO (Merck Portugal, Lisboa, Portugal). The kit for protein quantification was from Pierce (Biocontec, Lisboa, Portugal). The secondary alkaline phosphatase-conjugated anti-rabbit IgG detection kit (AP-10) and recombinant proteins, PKC-α (PK11), PKC-βI (PK16), PKC-δ (PK31), PKC-η (PK46) and PKC-ζ (PK41), were from Oxford Biomedical Research (LabClinics, Barcelona, Spain). Nitrocellulose membranes and all reagents for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and immunoblots were from BioRad (PACI, Lisboa, Portugal). Acid-washed glass beads, antibodies to PKC-α, PKC-βI, PKC-δ, PKC-η and PKC-ζ, aprotinin, arachidonic acid sodium salt, chelerythrine cloride, leupeptin, pepstatin A, phenylmethylsulfonyl fluoride, phorbol 12-myristate 13-acetate (PMA) were from Sigma Aldrich (Sintra, Portugal). All other chemicals used were of analytical grade.

Yeast Transformation and Cell Cultures

Constructed yeast expression plasmids YEp52 and YEp51, encoding the cDNA for bovine PKC-α and for rat PKC-βI, respectively (kindly provided by Dr. Heimo Riedel, Wayne University, Detroit, USA) and YEplac181, encoding the cDNA for the rat

PKC-δ, mouse PKC-η or PKC-ζ (kindly provided by Dr. Nigel Goode, Royal Veterinary College, London, UK) were amplified in *Escherichia coli* DH5α and confirmed by restriction analysis. The plasmids used contain galactose-inducible transcriptional elements and the leu2 gene for selection. *Saccharomyces cerevisiae* (*S. cerevisiae*; strain CG379; α *ade5 his7-2 leu2-112 trp1-289α ura3-52 [Kil-O]*; Yeast Genetic Stock Center, University of California, Berkeley, USA) was transformed using the lithium acetate method.[21] To ensure the selection of transformed yeast, cells were grown in leucine-free medium, in 1.5% agar plates, at 30°C.

For the yeast phenotypic assay, transformed cells were incubated in leucine free-medium, with slow shaking, at 30°C. The leucine free-medium contained 0.7% yeast nitrogen base, 2% glucose (w/v) or the indicated carbon source, amino acids, purines and pyrimidines, according to the transformed yeast requirements. Galactose (2%; w/v), instead of glucose, was included to induce transcription of the mammalian PKC gene.

Cell Lysis and Immunoblotting

Cell lysis was performed basically as described.[4] The protein concentration was determined using a kit for protein quantification (*Coomassie® Protein Assay Reagent Kit*, Pierce, Biocontec, Lisboa, Portugal). Similar amounts of protein (~40 μg) from protein extracts were then separated on 10% SDS-polyacrylamide gels (Mini-Protean II, BioRad, Hercules, CA, USA). Positive controls (4 μg) were obtained using recombinant proteins PKC-α (M_r 76,799 Da), PKC-βI (M_r 76,790 Da), PKC-δ (M_r 77,517 Da), PKC-η (M_r 77,600 Da) and PKC-ζ (M_r 67,740 Da). Proteins were electrophoretically transferred to nitrocellulose membranes and probed on immunoblots with specific rabbit antibodies to the individual mammalian PKC isoforms and revealed with a secondary alkaline phosphatase-conjugated anti-rabbit IgG (AP-10, Oxford Biomedical Research, LabClinics, Barcelona, Spain).

Yeast Phenotypic Assay

Transformed yeast cultures were incubated in leucine-free medium. Optical density values, measured at 620 nm (OD_{620}; Cary 1E Varian spectrophotometer, Palo Alto, CA, USA), were used as an indicator of growth. Transformed yeast were grown to an OD_{620} of approximately 1, collected by centrifugation and diluted to an OD_{620} of 0.05, in medium containing 2% (w/v) galactose (gene transcription-inducer) and 3% (v/v) glycerol (alternative carbon source). Diluted cultures (200 μl) were transferred to 96-wells microtitre plates and incubated for up to 100 h, with slow shaking at 30°C, either in the presence of drugs or solvent (DMSO 0.1%; final concentration). Growth was monitored by determining the OD_{620} using a plate reader (BioRad Benchmark Microplate Reader; Hercules, CA, USA). In preliminary experiments, growth curves for individual isoforms were determined and the duration of the logarithmic and stationary phases identified. Estimation of drug effects was based on OD_{620} measurements at fixed time points (at 65 h for cPKC isoforms or at 48 h incubation for nPKC and aPKC), times occurring during the respective logarithmic phase and where a "steady-state growth inhibition" (period of time during which maximal inhibition of growth was reached and remained constant or changed only slightly) was reached. In individual experiments, OD_{620} was routinely monitored for up to 100 h to confirm whether the "fixed time points" chosen were appropriate for all the compounds studied (PMA or arachidonic acid). The difference between the maximal OD_{620} reached and that measured at the beginning of incubation was used as an index of yeast growth. Drugs or solvent were added at the beginning and kept throughout the incubation. Yeast growth in the presence of drugs was expressed as percentage of growth observed in parallel experiments in the presence of solvent; it was further transformed into growth inhibition by subtracting that value from 100. Because growth inhibition caused by a maximal concentration of the standard PKC activator varied between isoforms, 100% growth inhibition was assumed to be that caused by 10^{-5} M PMA (or arachidonic acid for PKC-ζ), in order to standardise the maximal inhibition attainable; 0% growth inhibition would occur when growth in the presence of drugs was identical to that in the presence of solvent. Effects of PKC activators were expressed as a percentage of that effect.

For interaction experiments, a single concentration of PKC inhibitor chelerythrine or kielcorins **1–5** was added to PMA (10^{-8}–10^{-5} M; arachidonic acid for PKC-ζ). Concentration-response curves for the PKC activator in the absence or in the presence of chelerythrine or kielcorins **1–5**, were obtained and the concentration of PKC activator that caused 50% growth inhibition (EC_{50}) calculated. For each compound the EC_{50} ratios [EC_{50} (PKC activator + compound)/EC_{50} (PKC activator)] were calculated on each PKC isoform tested.

In Vitro Kinase Assay

The direct interaction between the compounds tested (PKC inhibitor chelerythrine and kielcorins **1–5**) and PKC was studied *in vitro* using the IQ™ PKC Assay Kit—Pseudosubstrate Peptide Substrate from Pierce Biotechnology (PACI, Lisboa, Portugal).

The assay was performed according to the manufacturer instructions for the 96-well plate format. Kielcorins **1–5** and the standard PKC inhibitor chelerythrine were tested at the concentration of 10^{-8} M. Purified PKC enzyme standard from rat brain (containing a mixture of α, β and γ isoforms) was from Pierce Biotechnology (PACI, Lisboa, Portugal); it was used at a final concentration of 0.01 U. Reaction mixtures containing the endogenous PKC activator phosphatidyl-L-serine, were incubated for 90 min and fluorescence intensity determined using a microplate spectrofluorometer SPECTRAmax®GEMINI XS (Molecular Devices Corporation, Sunnyvale, CA, USA). In the presence of PKC, there was a decrease in relative fluorescence units (RFU), reflecting phosphorylation of the fluorescent substrate. In the presence of PKC inhibitors, substrate phosphorylation is reduced causing an increase in RFU. RFU in samples not containing PKC were considered as 100%. Effects of drugs were expressed as a percentage of that value.

Statistical Analysis

Results are given as arithmetic means ± SEM and *n* represents the number of determinations. Differences between means were tested for significance using either paired Student's *t* test and unpaired Student's *t* test. A *P* value less than 0.05 was taken to be statistically significant.

RESULTS

Expression of mammalian PKC-α, -βI, -δ, -η and -ζ was confirmed by immunoblotting, using protein extracts of yeast cells, transformed with the plasmid containing the gene for a single mammalian PKC isoform, grown in the presence of the transcription inducer (2% galactose). Expression of each of these PKC isoforms resulted in a single antigenic band, which co-migrated with the respective recombinant protein. Protein extracts of transformed yeast cells grown in the absence of galactose did not present antigenic bands (Figure 4).

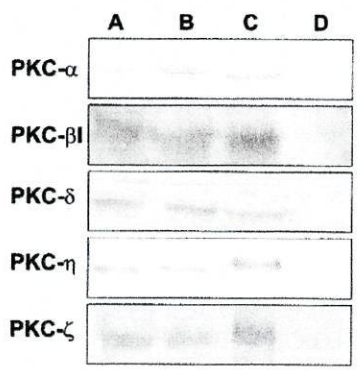


FIGURE 4 Immunodetection of PKC-α, -βI, -δ, -η and -ζ isoforms expressed in transformed *Saccharomyces cerevisiae* (CG379). Individual immunoblots are presented in a horizontal arrangement and were obtained from proteins extracts (~40 μg protein/lane) from cultures grown in selective medium in the presence of 2% galactose (lanes A and B; duplicate samples) or in the absence of 2% galactose (lane D). Positive controls (lane C; 4 μg) were obtained using recombinant proteins PKC-α (M_r 76,799 Da), PKC-βI (M_r 76,790 Da), PKC-δ (M_r 77,517 Da), PKC-η (M_r 77,600 Da) and PKC-ζ (M_r 67,740 Da).

Yeast Phenotypic Assay

PMA (considered a standard activator for the classical and novel PKC isoforms) was tested in yeast expressing PKC-α, -βI, -δ or -η, in concentrations up to 10^{-5} M (higher concentrations were not possible to test due to its low solubility in the culture medium). Since atypical PKC isoforms are not activated by phorbol esters,[22] arachidonic acid (up to 10^{-5} M) was used as the standard activator for PKC-ζ.[23] Maximal values of growth inhibition caused by 10^{-5} M PMA (or arachidonic acid for PKC-ζ), on the PKC isoforms tested are presented in Table I and were assumed to correspond to 100% growth inhibition. Effects of drugs were expressed as percentage of that maximal growth inhibition.

In the absence of galactose, neither PMA nor arachidonic acid altered yeast growth. The solvent used (DMSO; final concentration 0.1%) did not change yeast growth either in the presence or in the absence of galactose (not shown).

The influence of the kielcorin derivatives: *trans*-(±)-kielcorin C (**1**), *cis*-(±)-kielcorin C (**2**), *trans*-(±)-kielcorin D (**3**), *trans*-(±)-isokielcorin D (**4**) and *trans*-(±)-kielcorin E (**5**) (Figure 1) and of the established PKC inhibitor chelerythrine (Figure 3) on the growth of yeast expressing individual PKC isoforms was studied. In the absence of galactose, chelerythrine and kielcorins **1–5**, all tested at a concentration of 10^{-5} M, did not influence yeast growth.

In the presence of galactose, PMA caused a concentration-dependent inhibition of growth of yeast expressing cPKCs or nPKCs isoforms.

TABLE I Yeast growth inhibition caused by 10^{-5} M PMA on the PKC isoforms studied

PKC isoforms	Growth inhibition caused by 10^{-5} M PMA (% of control)
α	40.6 ± 1.9 (n = 36)
βI	36.1 ± 1.1 (n = 56)
δ	26.6 ± 0.6 (n = 52)
η	21.3 ± 0.7 (n = 52)
ζ	0.2 ± 1.4 (n = 36)[a]

[a] Growth inhibition caused by 10^{-5} M arachidonic acid was 23.9 ± 0.7 (n = 56). Growth in the presence of solvent was considered to be 0% growth inhibition (100% growth; see Experimental for details). Each value represents means ± SEM of the indicated n determinations.

EC_{50} values (nM) were, for the isoform indicated, 99.4 ± 10.1 (PKC-α), 214.7 ± 48.0 (PKC-βI), 453.1 ± 32.8 (PKC-δ) and 7.6 ± 0.6 (PKC-η) ($n = 64$). In yeast expressing PKC-ζ, arachidonic acid, but not PMA, caused a concentration-dependent growth inhibition of yeast expressing this isoform, with an EC_{50} of 208.2 ± 30.3 nM ($n = 64$).

In the yeast phenotypic assay, a PKC inhibitor blocks the growth inhibition caused by a PKC activator.[8,9] Therefore, the effects of chelerythrine and kielcorins **1–5** on growth inhibition caused by the appropriate PKC activator (PMA for PKC-α, βI, -δ and -η; arachidonic acid for PKC-ζ) were investigated.

In a first approach, chelerythrine and kielcorins **1–5** were tested alone. At the maximal concentration feasible under these experimental conditions (10^{-5} M), chelerythrine and kielcorins **1–5** caused, *per se*, stimulation of growth of yeast expressing individual mammalian PKC isoforms. A decrease in the concentration reduced this growth stimulation and, at a concentration of 10^{-8} M, chelerythrine and kielcorins **1–5** failed to cause a significant stimulation of yeast growth (data not shown).

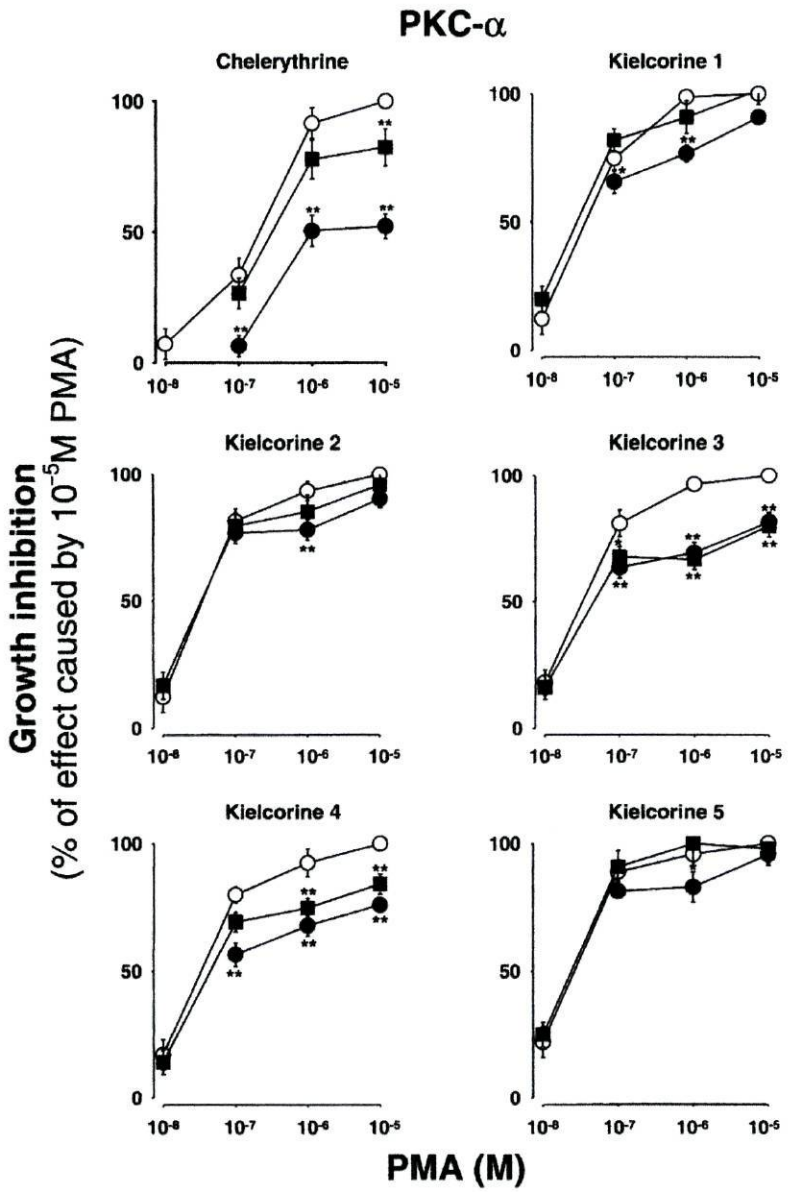


FIGURE 5 Interaction experiments on PKC-α: concentration-response curves for PMA alone (open circles) and in the presence of 10^{-5} M (filled circles) or 10^{-8} M (filled squares) of chelerythrine or kielcorins 1–5. Results are expressed as % of the maximal effect caused by 10^{-5} M PMA. Shown are means ± SEM of 16 – 20 determinations. Significantly different from growth inhibition caused by PMA alone: $^{*}P < 0.05$, $^{**}P < 0.001$ (unpaired Student's t test).

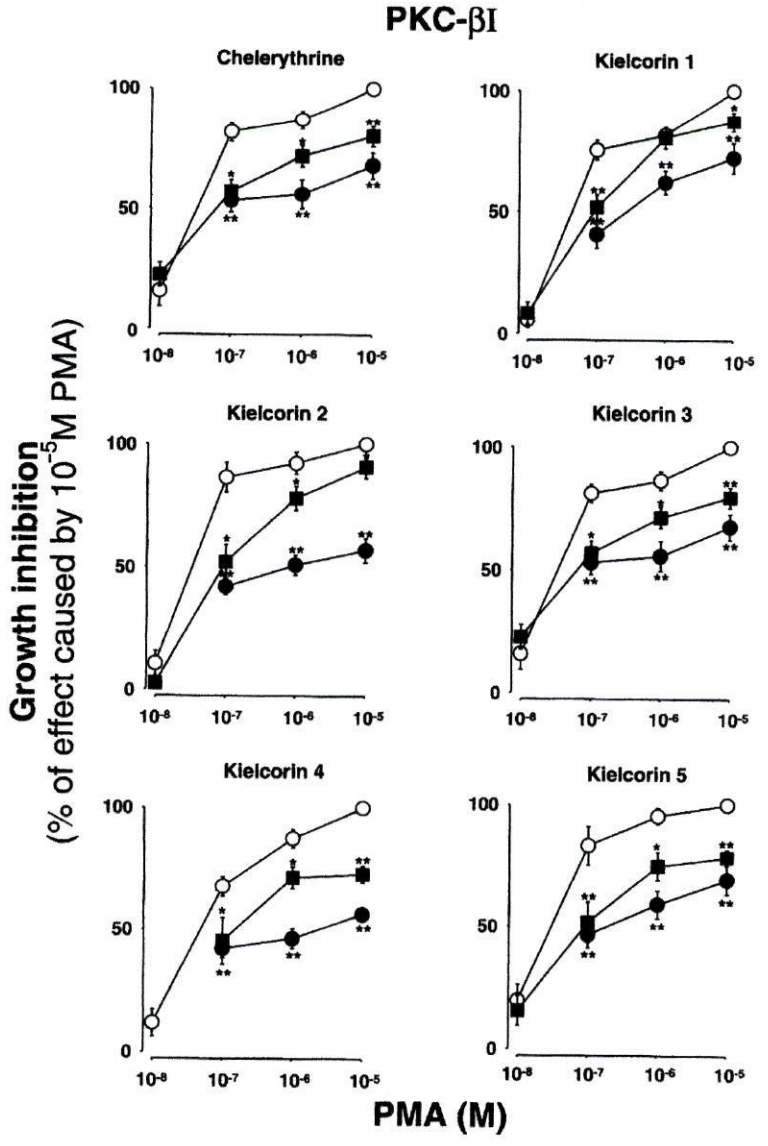


FIGURE 6 Interaction experiments on PKC-βI: concentration-response curves for PMA alone (open circles) and in the presence of 10^{-5}M (filled circles) or 10^{-8}M (filled squares) of chelerythrine or kielcorins **1–5**. Results are expressed as % of the maximal effect caused by 10^{-5}M PMA. Shown are means ± SEM of 16–20 determinations. Significantly different from growth inhibition caused by PMA alone: $^{*}P < 0.05$. $^{**}P < 0.001$ (unpaired Student's *t* test).

In a subsequent series of experiments, the ability of chelerythrine and kielcorins **1–5** to block growth inhibition caused by the appropriate PKC activator, was investigated. Chelerythrine and kielcorins **1–5** were tested at the maximal concentration feasible (concentration at which a stimulation of yeast growth was observed) and at 10^{-8}M (concentration at which no stimulation of yeast growth was observed). Effects of chelerythrine and kielcorins **1–5** on the growth inhibition caused by the PKC activator PMA on yeast expressing PKC-α, -βI, -δ or -η are shown in Figures 5,6,7 and 8, respectively. Figure 9 shows effects of chelerythrine and kielcorins **1–5** on the growth inhibition caused by arachidonic acid on yeast expressing PKC-ζ.

Chelerythrine reduced growth inhibition caused by the PKC activator (PMA or arachidonic acid), causing a shift to the right for the concentration-response curve of the PKC activator on all isoforms tested (compare open and filled symbols in left upper panel of Figures 5,6,7,8 and 9). This shift to the right was concentration-dependent, being the displacement caused by 10^{-8}M chelerythrine which was less pronounced than that caused by 10^{-5}M (compare filled squares and circles in left upper panel of Figures 5,6,7,8 and 9). In general, kielcorins also caused a shift to the right of the concentration-response curve for the PKC activator (compare open and filled symbols of Figures 5,6,7,8 and 9). The main exception occurred with PKC-α, where kielcorins **1, 2**

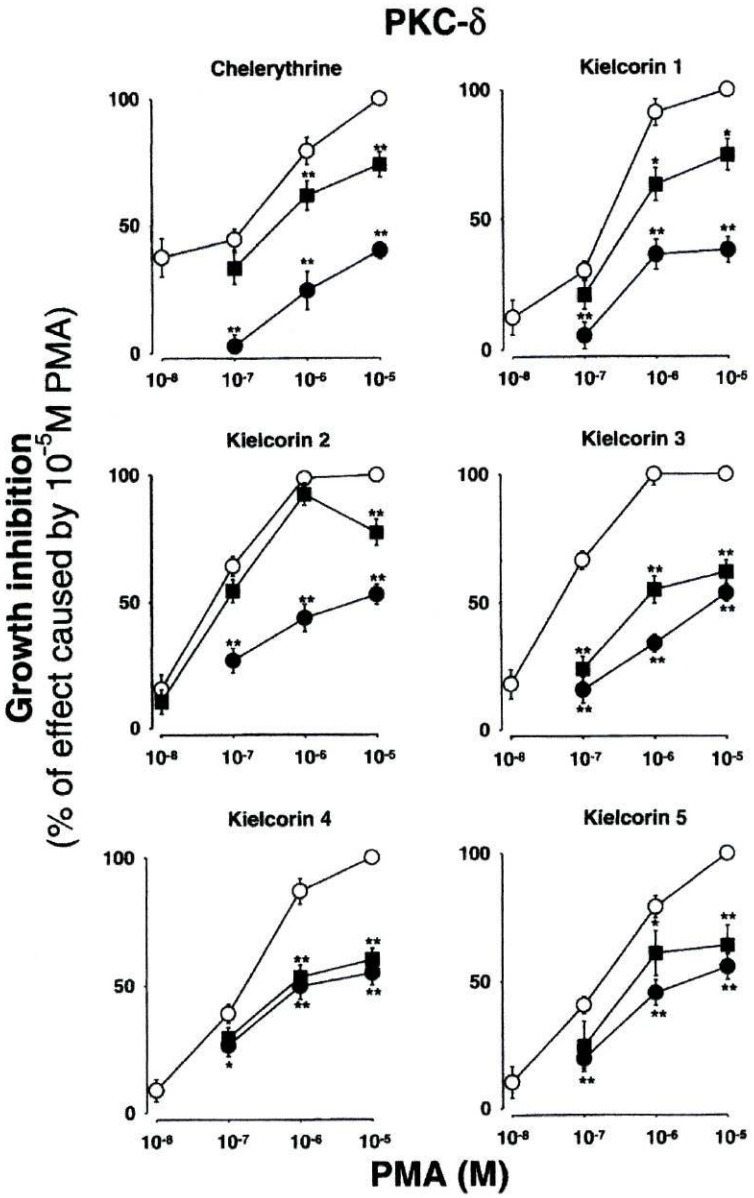


FIGURE 7 Interaction experiments on PKC-δ: concentration-response curves for PMA alone (open circles) and in the presence of 10^{-5} M (filled circles) or 10^{-8} M (filled squares) of chelerythrine or kielcorins **1–5**. Results are expressed as % of the maximal effect caused by 10^{-5} M PMA. Shown are means ± SEM of 16–20 determinations. Significantly different from growth inhibition caused by PMA alone: $^{*}P < 0.05$, $^{**}P < 0.001$ (unpaired Student's *t* test).

and **5**, at 10^{-8} M, failed to significantly influence the concentration-response curve for PMA (compare open and filled symbols of Figure 5). The shift to the right caused by kielcorins was, in general, concentration-dependent, being the displacement caused by 10^{-8} M which was less pronounced than that caused by 10^{-5} M kielcorins **1–5** (compare filled squares and circles of Figures 5,6,7,8 and 9). Exceptions occurred for kielcorin **5** on PKC-βI, kielcorins **4** and **5** on PKC-δ and kielcorin **3** on PKC-ζ, where no concentration-dependence was detected; in the presence of the highest and lowest concentration of these kielcorins the responses to the PKC activator were similar ($P > 0.05$; unpaired Student's *t* test).

In order to compare the potencies of chelerythrine and kielcorins **1–5** to block the growth inhibition caused by the appropriate PKC activator, EC_{50} values (*i.e.* the concentration of the PKC activator that caused half of the growth inhibition caused by 10^{-5} M PMA; arachidonic acid for PKC-ζ), obtained in the absence and in the presence of 10^{-8} M chelerythrine or kielcorins **1–5**, were calculated. When EC_{50} values, obtained in the presence of 10^{-8} M chelerythrine or one of the kielcorins **1–5**, were different from the EC_{50} values obtained in their

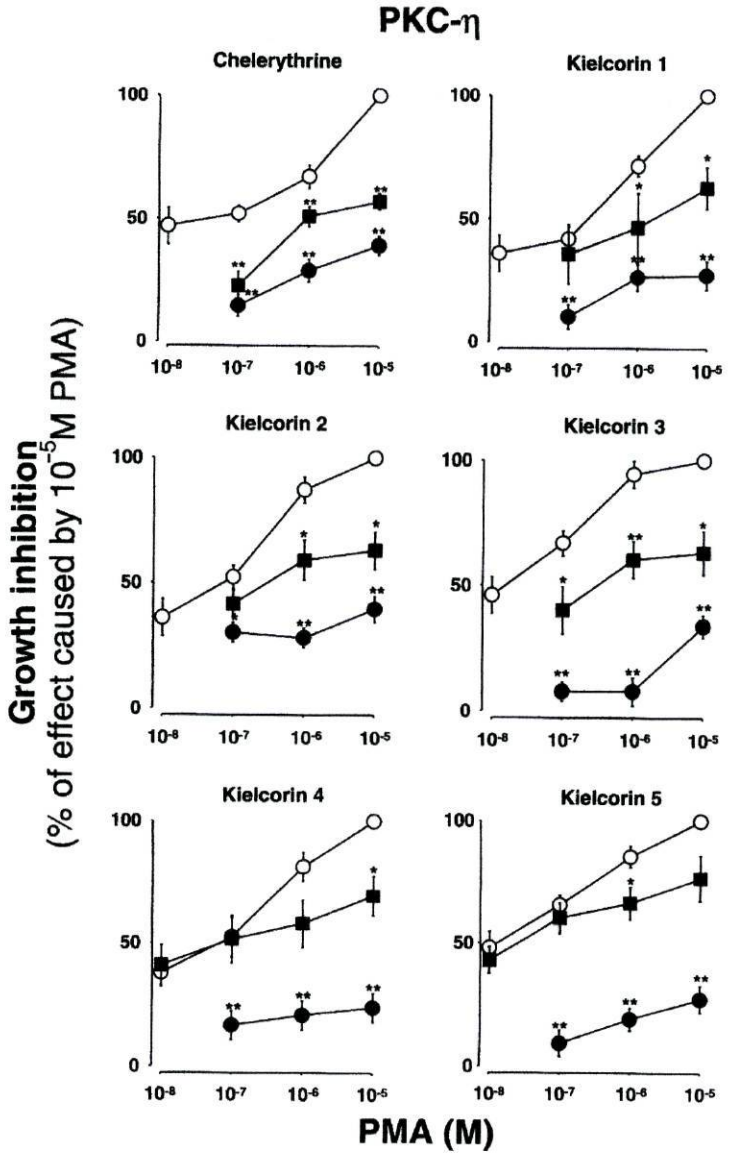


FIGURE 8 Interaction experiments on PKC-η: concentration-response curves for PMA alone (open circles) and in the presence of 10^{-5} M (filled circles) or 10^{-8} M (filled squares) of chelerythrine or kielcorins **1–5**. Results are expressed as % of the maximal effect caused by 10^{-5} M PMA. Shown are means ± SEM of 16–20 determinations. Significantly different from growth inhibition caused by PMA alone: $^{*}P < 0.05$, $^{**}P < 0.001$ (unpaired Student's *t* test).

absence, EC_{50} ratios were calculated. EC_{50} ratios were used to compare: *i*) the potency of kielcorins with that of chelerythrine and *ii*) the potencies of chelerythrine and kielcorins **1–5** on the different mammalian PKC isoforms individually expressed. Table II presents the EC_{50} ratios obtained with 10^{-8} M chelerythrine and kielcorins **1–5** (EC_{50} ratios obtained with 10^{-5} M were not calculated, because of the growth stimulation caused by chelerythrine and kielcorins **1–5**, *per se*).

Chelerythrine and kielcorins **1–5** presented differences in potencies towards the distinct PKC isoforms tested, as estimated by comparison of the respective EC_{50} ratios. The following order of potencies for the compounds studied have been established: chelerythrine, PKC $-\eta > -\beta I > -\alpha > -\zeta = -\delta$; kielcorin **1**, PKC $-\zeta > -\eta > -\delta = -\beta I$; kielcorin **2**, PKC $-\zeta > -\eta > -\beta I = -\delta$; kielcorin **3**, PKC $-\zeta = -\eta = -\delta$; kielcorin **4**, PKC $-\zeta > -\delta = -\beta I$; kielcorin **5**, PKC $-\zeta = -\beta I = -\delta$ (for definition of the rank order of potency, the $>$ was applied only when the EC_{50} ratio of the isoform placed at the left was significantly higher than that of the EC_{50} ratio placed at the right of the sign; otherwise $=$ was applied; $P < 0.05$; unpaired Student's *t* test).

When compared to chelerythrine, kielcorins **1–5** were shown to be less potent on PKC-α, failing, at the concentration tested, to change the PMA EC_{50}

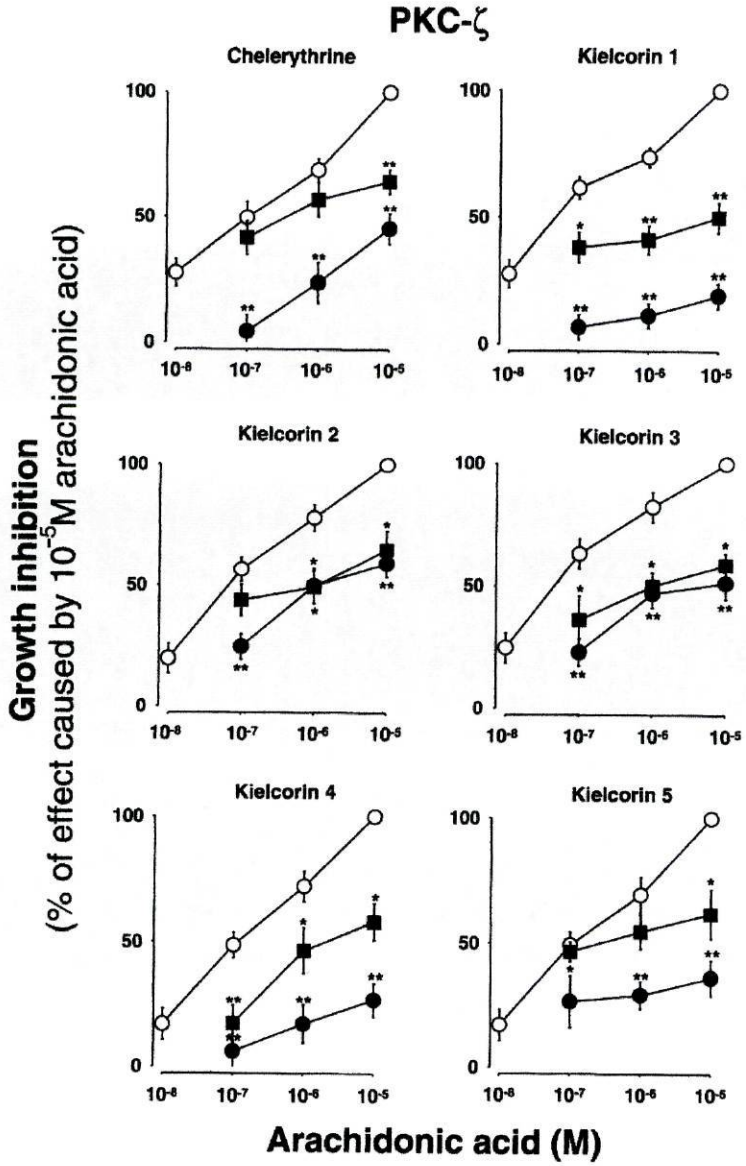


FIGURE 9 Interaction experiments on PKC-ζ: concentration-response curves obtained for arachidonic acid alone (open circles) and in the presence of 10^{-5}M (filled circles) or 10^{-8}M (filled squares) of chelerythrine or kielcorins **1–5**. Results are expressed as % of the maximal effect caused by 10^{-5}M of arachidonic acid. Shown are means ± SEM of 16–20 determinations. Significantly different from growth inhibition caused by arachidonic acid alone: $^{*}P < 0.05$, $^{**}P < 0.001$ (unpaired Student's *t* test).

TABLE II EC_{50} ratios for chelerythrine and kielcorin derivatives on the individual PKC isoforms tested

Compound	EC_{50} Ratio[a]				
	PKC-α	PKC-βI	PKC-δ	PKC-η	PKC-ζ
Chelerythrine	1.9 ± 0.3	2.9 ± 0.3*	1.3 ± 0.1	63.2 ± 1.7*	1.4 ± 0.2
Kielcorin **1**	*ND*	1.8 ± 0.5*	2.2 ± 0.6*	9.8 ± 1.7*†	213.0 ± 20.8†
Kielcorin **2**	*ND*	2.8 ± 0.7*	1.6 ± 0.3*	4.9 ± 0.6*†	17.9 ± 6.4†
Kielcorin **3**	*ND*	*ND*	12.6 ± 4.1†	24.7 ± 6.2†	25.1 ± 6.6†
Kielcorin **4**	*ND*	3.2 ± 0.9*	5.2 ± 0.9*†	*ND*	16.7 ± 5.2†
Kielcorin **5**	*ND*	2.6 ± 0.6	2.0 ± 0.8	*ND*	3.4 ± 0.8†

[a] EC_{50} ratio = EC_{50} (PKC activator + 10^{-5}M compound)/EC_{50} (PKC activator). The EC_{50} values were considered the concentration of PKC activator that caused half of the growth inhibition caused by 10^{-5}M of PMA (arachidonic acid for PKC-ζ). Shown are means ± SEM of 16–20 determinations. *ND*: Not determined (identical EC_{50} values in the absence or in the presence of the compound). Significant differences: from PKC-ζ, $^{*}P < 0.05$; from chelerythrine, $^{\dagger}P < 0.05$ (unpaired Student's *t* test).

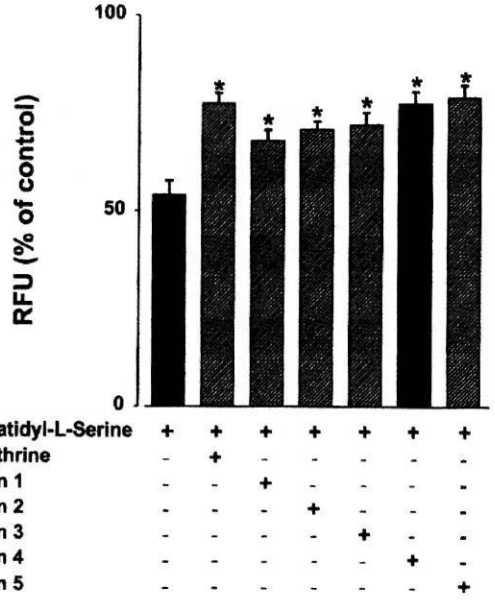


FIGURE 10 *In vitro* kinase assay using a purified rat brain PKC enzyme: effects of chelerythrine, and kielcorins **1**–**5** (all tested at the concentration of 10^{-8} M). Relative fluorescence units (RFU) were measured in samples without enzyme (100%; control) or with PKC, which caused a decrease in RFU (see Experimental for details). Phosphatidyl-L-serine, the recommended PKC activator, was present in all samples; drugs or solvent, were tested in the absence or in the presence of PKC. Effects of drugs were expressed as % of RFU obtained in the absence of PKC. Shown are means ± SEM of 4 assays (8 assays for phosphatidyl-L-serine alone). Significantly different from solvent (phosphatidyl-L-serine alone): $^{*}P < 0.05$ (one way ANOVA, followed by Tukey's *post-hoc* test).

value. On PKC-βI, kielcorin **3** also did not change the PMA EC_{50}, but kielcorins **1**, **2**, **4** and **5** were shown to be as potent as chelerythrine. On PKC-δ, kielcorins **1**, **2** and **5** were shown to be as potent as chelerythrine, whereas kielcorins **3** and **4** were more potent than chelerythrine, kielcorin **3** having a much higher potency. On PKC-η, kielcorins **4** and **5** failed to alter the PMA EC_{50}, whereas kielcorins **1**, **2** and **3** markedly inhibited PMA effects, although less than chelerythrine, which on this PKC isoform showed its highest potency. On PKC-ζ, all kielcorins were more potent than chelerythrine, kielcorin **1** having an extraordinary high potency when compared with those of other kielcorins or chelerythrine.

In Vitro Kinase Assay

To test whether kielcorins **1**–**5** were interacting directly with PKC, their effects were studied *in vitro*, using purified rat brain PKC. The assay used is based on the ability of PKC inhibitors to reverse the fluorescence intensity quenching (a decrease on the relative fluorescence units, RFU) caused by the PKC activator (phosphatidyl-L-serine as recommended by the method). Reversing of fluorescence quenching (which reflects a reduction of substrate phosphorylation) is assumed to be an indicator of inhibition of PKC catalytic activity. Kielcorins **1**–**5** and chelerythrine were used at the lowest concentration tested (10^{-8} M), the same used to obtain the EC_{50} ratios presented in Table II. Kielcorins **1**–**5** were able to reverse the effect of the endogenous PKC activator. The effects elicited by these compounds were similar to that caused by the PKC inhibitor chelerythrine (Figure 10).

Computational Studies

The optimised geometries of kielcorins **1**–**5** and chelerythrine were calculated and their potential isosurfaces obtained and these are shown in Figure 11. All kielcorins tested (**1**–**5**) contain three main negative potential points, namely, one over the carbonyl of the xanthonic nucleus, another near the hydroxymethyl group and the third located over

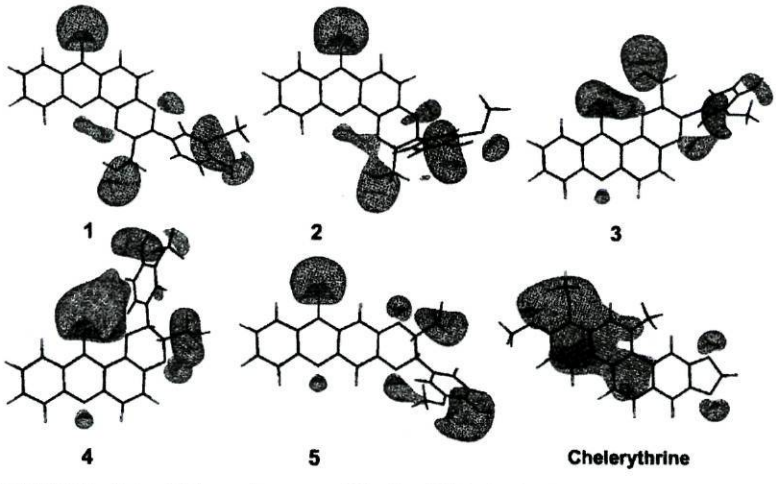


FIGURE 11 Potential isosurfaces at -20 kcal mol^{-1} for kielcorins **1**–**5** and chelerythrine.

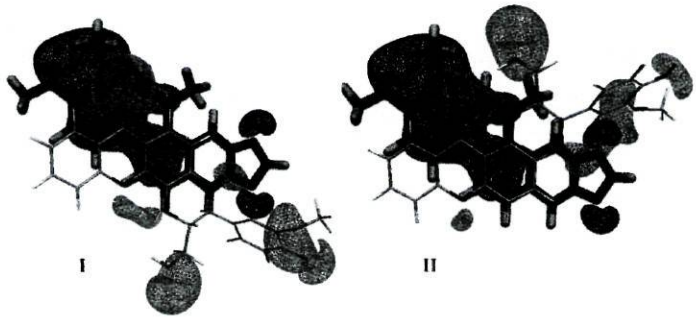


FIGURE 12 Two examples of correspondence of negative potential surfaces: (I) chelerythrine and kielcorin **1**; (II) chelerythrine and kielcorin **3**.

the oxygenated substituents of the aromatic ring in the coniferyl unit. For kielcorins **3** and **4**, the negative potential point over the carbonyl is reinforced by the proximity of the other two regions. Concerning chelerythrine, a strong negative potential over the aromatic area and two others over the methylenedioxy group can be noted. A correspondence of the potential points referred to above for the kielcorins **1–5** with chelerythrine was achieved (Figure 12). For kielcorins **3–5** the correspondence of the potential bulk of the carbonyl group with the aromatic region of chelerythrine, allows a further correspondence among the other potential points. Figure 12 shows the common features of the negative potential surfaces with chelerythrine, for kielcorin **1** (with a very high potency on PKC-ζ) and also for kielcorin **3** (the most potent on PKC-δ and, like chelerythrine, with a high potency on PKC-η).

DISCUSSION

The yeast phenotypic assay, which uses transformed yeast expressing an individual mammalian PKC isoforms, has been proposed as a rapid and simple assay for the screening of potential PKC modulators. In the present study, either mammalian PKC-α, -βI, -δ, -η or -ζ isoforms were expressed using the same yeast strain. According to previous studies,[6,7] exposure of transformed yeast expressing a mammalian PKC isoform to a PKC activator, led to a concentration-dependent inhibition of yeast growth (reflecting an increase in the cell doubling time). Inhibition of transformed yeast growth caused by the PKC activator was assumed to be due to its interaction with the mammalian PKC isoform expressed because it did not occur when transformed yeast cells were incubated in medium lacking galactose, the expression inducer. Confirmation that mammalian PKC isoforms were expressed in the presence of galactose (and not on its absence) was obtained by immunobloting.

Absolute values of growth inhibition caused by the PKC activators used (PMA or arachidonic acid) differed among the expressed PKC isoforms. These differences can be ascribed to different levels of expression of the mammalian PKC isoform or to differences in the catalytic potential of the expressed isoform. The latter possibility is supported by results obtained *in vitro* that showed that identical amounts of PKC isoforms, in the presence of the same concentration of a PKC activator, led to different degrees of phosphorylation of a given substract.[24] In order to avoid the influence of different absolute values on data interpretation, growth inhibition caused by PKC activators was standardised. For each isoform, growth inhibition caused by a maximal concentration of PKC activator was assumed to be the 100% of growth inhibition attainable by PKC activators under the present experimental conditions and EC_{50} values estimated from the concentration-response curves obtained.

Chelerythrine and kielcorins **1–5**, when tested in the micromolar range, cause a slight stimulation of growth of transformed yeast. This effect was observed only when a mammalian PKC isoform was expressed and, therefore seems to result from an interaction with the expressed mammalian PKC isoform. A direct stimulation of growth of transformed yeast has been reported previously[8] and may result from blockade of an endogenous activation of PKC.

Blockade of PKC activator-induced growth inhibition by established PKC inhibitors has been reported to reflect inhibition of the mammalian PKC isoform expressed.[8,9] Under these conditions, PKC inhibitors caused a shift to the right of the concentration-response curve of the PKC activator. When this occurred, the ratio between the PKC activator EC_{50} values (obtained in the presence and in the absence of the PKC inhibitor) was used as an index of the PKC inhibitor potency on the mammalian PKC isoform expressed. To avoid putative influences on the shift to the right caused by the growth stimulation observed with the highest

concentration of chelerythrine and kielcorins, their potencies were compared by the shift caused by 10^{-8} M (same concentration for all the compounds tested). As expected, chelerythrine inhibited effects of the PKC activators on growth of transformed yeast, being active on all isoforms tested. Kielcorins also inhibited effects of PKC activators. Kielcorins were particularly potent on PKC-ζ and showed with PKC-α their lowest potency. Kielcorins were less potent than chelerythrine on PKC-η, and were as potent as chelerythrine on PKC-βI. However, on PKC-δ, kielcorins **3** and **4** were more potent than chelerythrine, and on PKC-ζ, all kielcorins **1**–**5** were more potent than chelerythrine. Among the kielcorins tested, kielcorin **1** presented the most marked difference on potency, being particularly potent on PKC-ζ; the less marked differences were observed with kielcorin **5**. Kielcorin **3** presented a similar high potency on several isoforms (PKC-δ, -η and -ζ).

Confirmation that kielcorins blocked the effect of PKC activators on yeast growth by interacting with PKC was obtained using an *in vitro* kinase assay. At the lowest concentration used on the yeast phenotypic assay, kielcorins **1**–**5** inhibited PKC catalytic activity, an effect similar to that caused by the established PKC inhibitor chelerythrine.

The similarity of results obtained for chelerythrine and kielcorins, in the yeast phenotypic assay and in *in vitro* kinase assay, raised the possibility that they could have common electronic properties. Although PKC inhibitors are known for their large structural diversity,[25] common structural and electronic features were found among kielcorins **1**–**5** and chelerythrine (preliminary computational studies described in Results) which can explain the functional similarities detected between these two groups of compounds as PKC inhibitors.

This study is, to our knowledge, the first attempt to unravel the molecular basis for the biological activity of kielcorins, and suggests that kielcorin derivatives constitute a promising group from which to obtain new isoform selective PKC inhibitors.

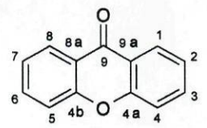
Acknowledgements

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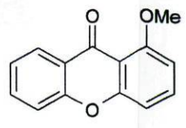
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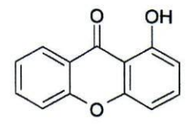
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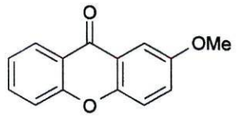
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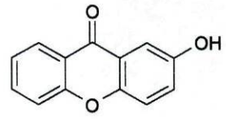
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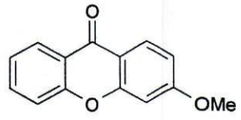
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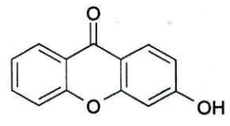
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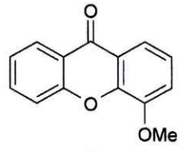
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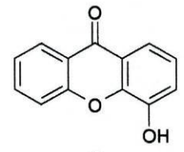
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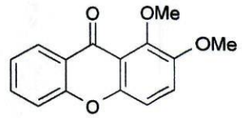
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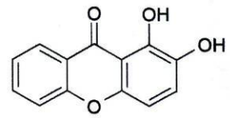
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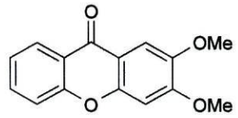
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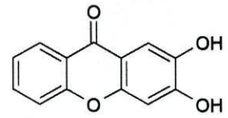
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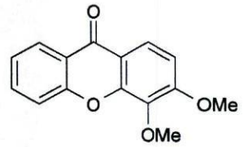
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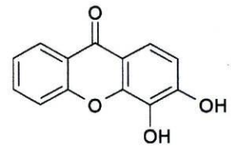
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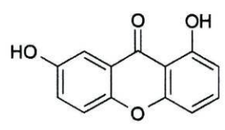
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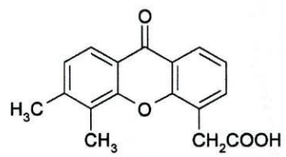
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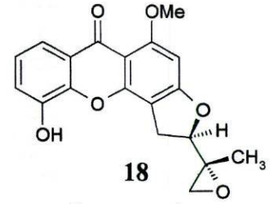
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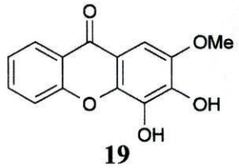
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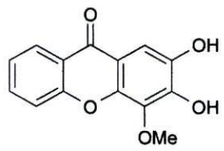
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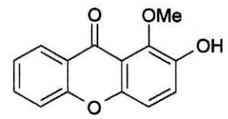
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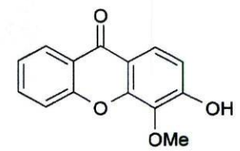
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3,4-Di-hidroxi-2-metoxixantona



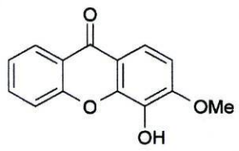
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2,3-Di-hidroxi-4-metoxixantona



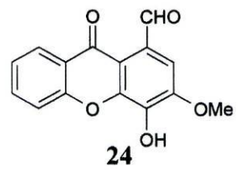
21
2-Hidroxi-1-metoxixantona



22
3-Hidroxi-4-metoxixantona

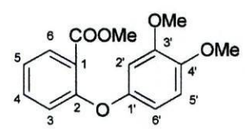


23
4-Hidroxi-3-metoxixantona

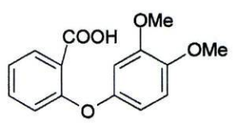


24
1-Formil-4-hidroxi-
-3-metoxixantona

ÉTERES BIFENÍLICOS

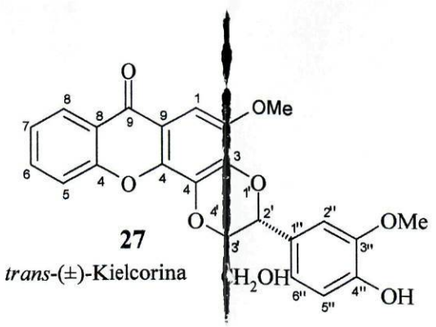


25
2-(3,4-Dimetoxifenoxi)-benzoato de metilo



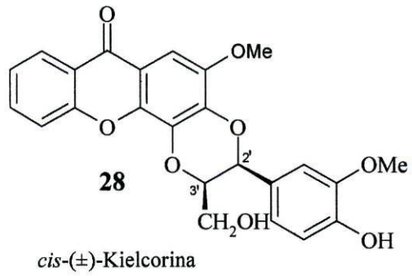
26
Ácido 2-(3,4-dimetoxifenoxi)-benzóico

XANTONOLIGNÓIDES



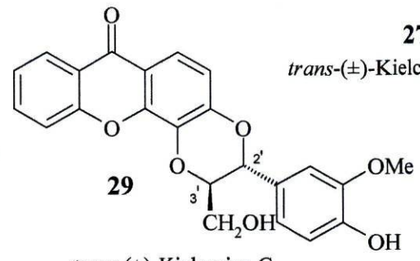
27

trans-(±)-Kielcorina



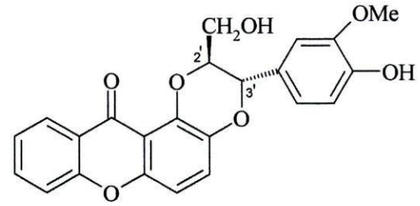
28

cis-(±)-Kielcorina



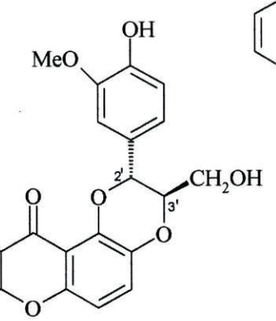
29

trans-(±)-Kielcorina C



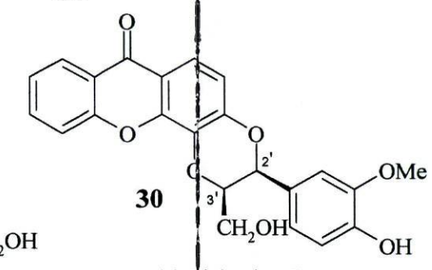
31

trans-(±)-Kielcorina D



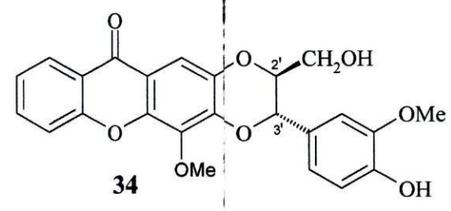
32

trans-(±)-Isokielcorina D



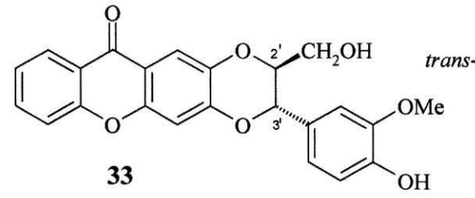
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cis-(±)-Kielcorina C



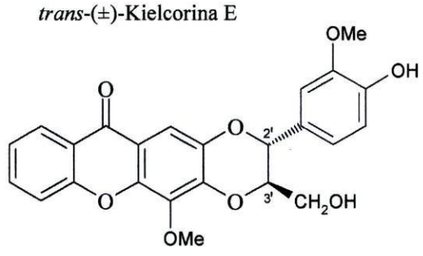
34

trans-(±)-Kielcorina B



33

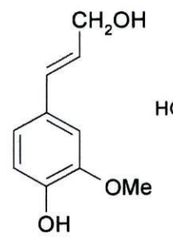
trans-(±)-Kielcorina E



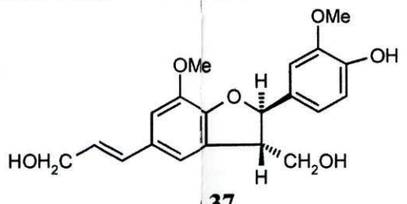
35

trans-(±)-Isokielcorina B

ÁLCOOL CONIFERÍLICO (34) E DERIVADOS



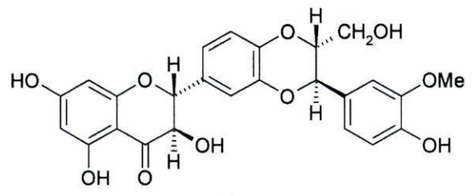
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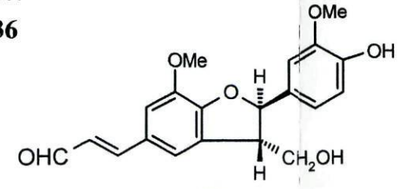
37

Álcool desidrodiconiferílico

SILIBINA



39



38

Fenilcumarano